EFFECT OF MATERNAL NUTRIENT RESTRICTION ON FETAL SKELETAL MUSCLE GROWTH AND METABOLISM IN SHEEP

A Dissertation

by

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ABSTRACT

Maternal nutrient restriction (NR) causes small for gestational age offspring (SGA), which exhibit a higher risk for metabolic syndrome. Skeletal muscle is among the most susceptible organs to maternal NR. Previous studies have typically considered NR fetuses as a single experimental group. However, using a sheep NR model, we routinely observe a spectral phenotype of fetal weights within the NR group, suggesting unique maternal-placental adaptations and fetal responses. Hence, studies were conducted to characterize particular aspects of fetal skeletal muscle growth and pathways for glucose uptake, while considering fetal weight variations within the NR group.

Singleton pregnancies (n=56) were generated by embryo transfer. Ewes received 100% (Control, n=12) or 50% (NR, n=44) of NRC nutrient requirements from gestational day (GD) 35 until necropsy at GD 135, when gastrocnemius and soleus muscles were collected. NR fetuses were separated into quartiles based on fetal weight, the highest (Non-SGA, n=11) and lowest (SGA, n=11) quartiles were selected for investigation. Results for muscle growth showed decreased cross-sectional area in both muscles within NR(SGA) group compared to controls, without alterations in pathways for protein synthesis (MTOR) or degradation (Glucocorticoid-KLF15-ubiquitin ligases) regardless of upregulation in *KLF15* (in gastrocnemius) and *BCAT2* (in soleus) in NR(SGA) and NR(Non-SGA) fetuses. NR(SGA) group had decreased total content of

amino acids, which suggests that lower availability of building blocks is a limiting factor for protein synthesis rate, without downregulating pathways involved in this process.

Results for glucose uptake pathways showed an increase of insulin and IGF1 receptors in response to a decrease of those hormones, and higher content of the glucose transporters SLC2A4 and SLC2A1 in NR(SGA) fetuses within gastrocnemius. These metabolic adaptations would support fetal survival while predisposing for metabolic dysregulations in adulthood. In both muscles, NR(SGA) group had lower type I myofiber proportion which if maintained postnatally would further affect metabolism.

Collectively, these results highlight that while most responses within NR(SGA) group are concordant with previous observations for SGA offspring, our NR(Non-SGA) group represents a different phenotype that is not entirely protected from NR programming, despite being of normal fetal weight. Results also identify muscle-specific responses to maternal NR.

DEDICATION

To the most important women of my life:

Melania del Carmen, my great grandmother

Rosa Elvira, my grandmother

Rosa Amelia, my mother

and

Carolina Alejandra, my sister

Thanks for your legacy

May your past experiences be the strengths of your future

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I always dreamed of having an academic degree from an institution of worldwide recognition. Today I can say that, with effort, dreams come true. Thanks to everyone who made it possible!

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Long-term maternal nutrient restriction (NR) during pregnancy impairs fetal growth, which leads to small for gestational age (SGA) offspring. This condition is associated with higher perinatal mortality and increased risk of metabolic syndrome during postnatal life (Barker et al., 1989). Skeletal muscle is among the most sensitive tissues to maternal NR, and it plays an essential role in metabolic dysregulation due to its prominence in glucose and oxidative metabolism (Brown, 2014). Additionally, the occurrence of SGA offspring in livestock species is associated with a decrease in productive performance, mostly in meat production, which depends on efficient skeletal muscle growth and metabolism. This becomes particularly relevant as current estimations suggest that worldwide protein production should be doubled by 2050 to meet the requirements of an increasing human population (Alexandriatos and Bruinsma, 2012). The situation becomes even more concerning when considering that hunger remains a severe problem in developing countries, in areas such as Africa and Asia (FAO, 2017).

The use of a livestock animal research model, such as the sheep, to understand the effect of maternal undernutrition in skeletal muscle growth and metabolism provides valuable information for translational research as well as insights to prevent inefficiencies in agricultural performance in the future.

1. Undernutrition and Low Birth Weight Prevalence in the World

Malnutrition has been defined by the World Health Organization (WHO) as any condition leading to overnutrition and obesity, famine and underweight, or dysregulation in intake of specific minerals and micronutrients (FAO, 2017). By 2017, more than one in every eight adults in the world were obese, and around one in nine were undernourished, which was estimated to represent a total of 821 million of undernourished people around the world. Famine and undernutrition cases have been continuously increasing since 2014 and are a public health concern primarily in developing and low-income countries (FAO, 2017). This is represented by the distribution of undernutrition in the world, where Africa represents 20.4%, Asia represents 11.4%, and Latin America and The Caribbean represent 6.1%, while less than 2.5% is represented by North American and Europe together (FAO, 2017).

The consequences of undernutrition are ample and include a higher predisposition for disease, and in extreme situations, death. This scenario becomes particularly challenging during periods of physiological increase of nutrient requirements; such as pregnancy since the female organism must support itself as well as placental and fetal growth. Maternal undernutrition during pregnancy results in SGA offspring, with more than 20 million cases reported annually (UNICEF, 2004). As expected, the distribution of low birth weight cases matches the geographical areas of adult undernutrition as 95.4% of the cases appear in developing countries, with most of them occurring in Asia and Africa, followed by lower incidence in Latin America and The Caribbean.

2. Maternal Nutrient Restriction and SGA Offspring

Low birthweight neonates include both, preterm births and infants born to term after experiencing intrauterine growth restriction. For humans, the concept of intrauterine growth restriction (IUGR) has been defined as the offspring placed below the 10^{th} percentile of fetal weight distribution at birth, and it is typically associated to asymmetric growth (Goldenberg and Cliver, 1997). In livestock species, maternal nutrient restriction is also a prevalent cause for IUGR, which have been defined as an impairment in gestational development of a fetus or its parts (Wu et al., 2006). A similar concept is small for gestational age (SGA) offspring, which is a broader classification and refers to fetuses that are smaller than expected for the species at a given gestational age (Goldenberg and Cliver, 1997). Individuals born as IUGR or SGA are more prone to suffer perinatal mortality and experience an increased risk for hypertension (Gennser et al., 1988), obesity (Fernandez-Twinn and Ozanne, 2006), type 2 diabetes (Rich-Edwards et al., 1999), heart disease (Barker et al., 1989) and metabolic syndrome (McMillen and Robinson, 2005).

Dr. David Barker was the first scientist to describe this correlation between maternal nutrient restriction, birth weight, and postnatal metabolic dysregulations. Based on studies conducted on adult offspring born to undernourished females during the Dutch famine, Hales and Barker (1992) stated the thrifty phenotype hypothesis. This hypothesis states that early life nutrient deficiency leads to a fetal programming effect that would support immediate survival and promote storage of nutrients, predominantly in the form of fat, rather than immediately using them (Gluckman et al., 2010). Nevertheless, during

postnatal life, these adaptations would lead to type 2 diabetes, obesity, and other dysregulations associated with metabolic syndrome (Symonds et al., 2009). The appearance of these conditions would be exacerbated by a postnatal scenario of normal, or excessive, nutrient availability. In this case there will be a mismatch between the prenatally anticipated environment and the actual postnatal conditions; consequently, the fetal adaptations to survive under prenatal nutrient scarcity will turn into negative health outcomes later in postnatal life (Gluckman et al., 2005; Hyatt et al., 2011).

3. Maternal Nutrient Restriction and Livestock Production

In addition to its effects on human health, maternal undernutrition also affects the production potential of livestock species (Wu et al., 2006). Animals that are usually raised based on grazing systems, such as sheep and cattle, are among the most susceptible to be affected due to low forage quantity and quality. Nowadays, climate change is a global factor that negatively impacts the natural environment (Walther et al., 2002) and has a direct detrimental effect on forage availability for livestock species. Alterations in rainy and drought periods, increase in desertification, and changes in temperature are likely to produce forage scarcity, and projections indicate that this situation will become even more severe in the future (Howden et al., 2008; Nardone et al., 2010).

This scenario of nutrient scarcity becomes even more critical under physiological conditions such as pregnancy, when the dam has to support its requirements, in addition to placental and fetal growth (Bauman and Currie, 1980). As a consequence, fetal growth is reduced in response to nutritional hardship, which in the context of livestock species brings negative outcomes in productive performance (Wu et al., 2006). For example,

SGA offspring is under a higher risk of neonatal death, which leads to economic losses for the industry. Impaired thermogenesis capacity is one of the causes of increased neonatal death in SGA neonates. Brown adipose tissue (BAT) plays an essential role in non-shivering thermogenesis, a primary mechanism for thermoregulation in the neonate (Satterfield and Wu, 2011). BAT development is impaired by maternal NR as demonstrated by a model of 50% NR in the sheep, which led to a decrease in fetal brown adipose tissue (BAT) weight (Satterfield et al., 2013). Interestingly, the study of Satterfield et al. (2013) found that arginine administration to 50% NR ewes was sufficient to rescue BAT growth to control levels, which would have a positive effect on neonate survival in fetuses from NR dams.

Additionally, animals born as SGA after prenatal nutrient restriction present a reduced efficiency in nutrient utilization (Wu et al., 2006) and experience an accelerated compensatory growth that favors adipose tissue deposition instead of skeletal muscle (De Blasio et al., 2007). This raises production costs and alters carcass composition towards increased fat and decreased lean mass (Ford et al., 2007), which reduces carcass value. Moreover, skeletal muscle development and growth are impaired by maternal nutrient restriction (Fahey et al., 2005; Quigley et al., 2005; Zhu et al., 2006; Costello et al., 2008; Brown, 2014) and this effect cannot be fully compensated during postnatal life because the number of muscle fibers is fixed before birth in livestock species (Picard et al., 2002). Hence, the capacity for lean mass deposition would be permanently diminished as a result of maternal NR, and meat production would be less efficient as a consequence.

A decrease in animal protein production as a result of maternal NR will have an impact on human nutrition and health because animal protein is an essential source of nutrients for the human population (Wu, 2016). Beyond its relevance in animal production, nutritional hardship in livestock species such as the sheep has also been recognized as a suitable model for translational research in fetal development (Barry and Anthony, 2008; Morrison, 2008). This allows for dual-purpose research with the potential to provide valuable knowledge for both fields, animal production, and human health and physiology.

4. Maternal Nutrient Restriction Effect on Fetal Organ Growth and Function – Sheep Model

The use of animal models for the study of fetal programming has provided supporting evidence for the initial epidemiological associations between maternal nutrient deprivation, SGA offspring, and metabolic alterations in postnatal life. Among different animal models, the sheep is a valuable alternative in an agricultural context, and for application into translational research, because it represents a close approximation to the level of maturity and organ development of a human fetus (Figure 1.1), it is a non-litter bearing species, and ovine fetuses are similar in size to human counterparts.

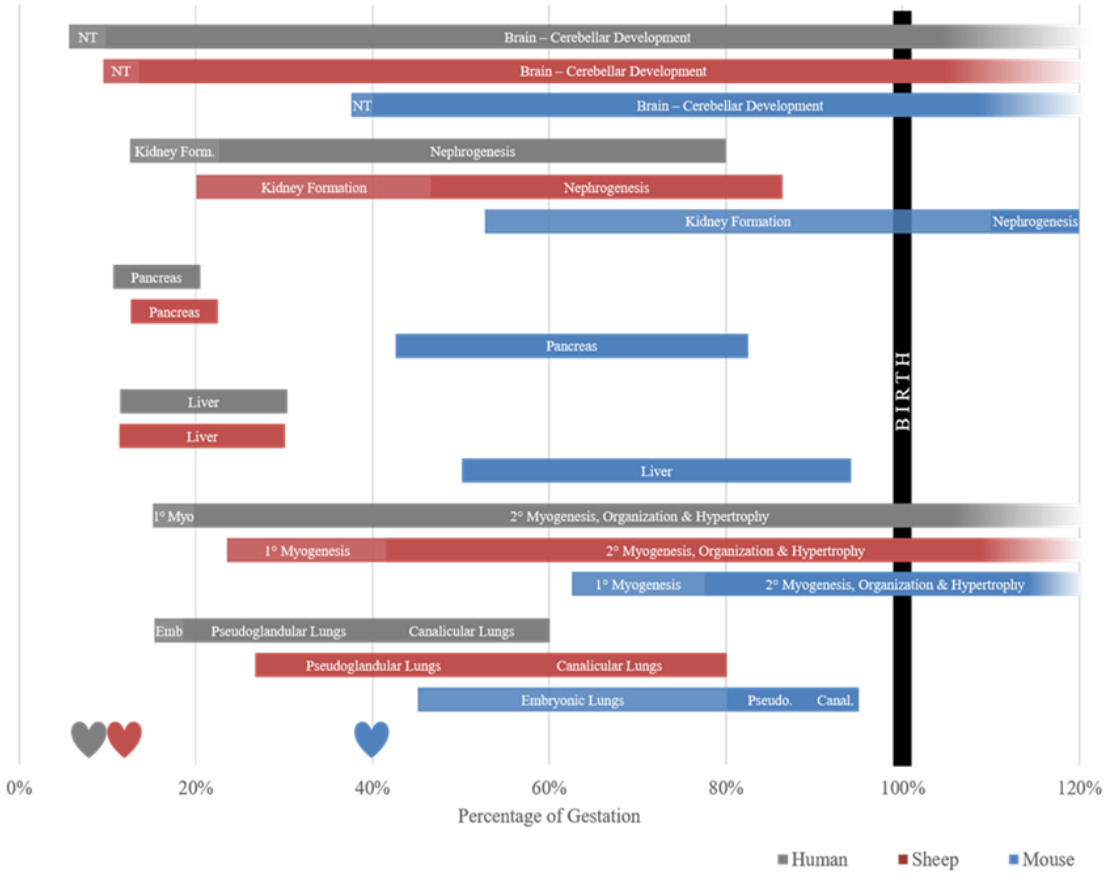

Figure 1.1 Comparative Organogenesis Timeline throughout Human, Sheep, and Mouse Gestation. Mouse offspring are born precocial and development of organs such as muscle and kidney is completed postnatally. When compared to the human timeline for development, the sheep represents a closer approximation than mouse. Both kidney and muscle development are completed prenatally in the sheep and human. Prenatal alterations in either kidney or muscle development would have the potential to be corrected postnatally in the mouse, while this potential will be low in sheep and human. A similar developmental timing is beneficial when translational research is the goal of fetal programming studies. **NT** (neural tube), **1° Myo** (primary myogenesis of skeletal muscle), **Emb** (embryonic lung development), **Pseudo** (pseudoglandular lung), **Canal** (canalicular lungs). Hearts represent first detectable heart beat (Sandoval et al., 2018).

Several studies have been conducted in the sheep to evaluate the effect of maternal NR in fetal organ growth and metabolism. A decrease in fetal weight has been a seminal finding of these studies (Osgerby et al., 2002; Kwon et al., 2004; Gao et al., 2008; Lassala et al., 2010; Satterfield et al., 2009; Satterfield et al., 2013). The study of Kwon et al. (2004) showed that decreased fetal growth in response to maternal NR was associated to a reduced concentration of polyamines and amino acids (branched-chain amino acids, serine, and arginine) in maternal and fetal plasma, as well as fetal allantoic and amniotic fluid. Realimentation of NR females from gestational day (GD) 78 to term was shown to prevent impaired fetal growth in this study (Kwon et al., 2004). Interestingly, the study of Lassala et al. (2010) found that decreased fetal weight in response to maternal NR could be prevented by administration of arginine from GD 60 to term, which further reinforces the relevance of amino acid availability for normal fetal growth.

Also, lower circulating levels of insulin like growth factor 1 (IGF1) and insulin have been reported in fetal plasma as a consequence of maternal NR (Osgerby et al., 2002). Both IGF1 (Baker et al., 1993) and insulin (Fowden et al., 1989) play an essential role in the stimulation of fetal growth.

Growth and metabolism in several fetal organs are affected by maternal NR in the sheep. Examples of these findings are ventricular hypertrophy in fetal heart (Vonnahme et al., 2003), impaired vasodilation capacity in coronary arteries (Shukla et al., 2014); and decreased glomeruli dotation (Brenner et al., 1988) and upregulated apoptosis (Lloyd et al., 2012) in fetal kidney. Also, reduction in weight of fetal pancreas

(Osgerby et al., 2002) and brown adipose tissue (Satterfield et al., 2013) have been described. Interestingly, pancreatic and brown adipose tissue growth was increased after maternal arginine administration (Satterfield et al., 2013). For liver, increased relative weight has been found at the fetal stage by GD 78 (Vonhamme et al., 2003), while decreased liver weight accompanied by increased expression in apoptotic factors was found in aged offspring after prenatal NR (Hyatt et al., 2007). Increased expression of PEPCK, a rate-limiting enzyme for gluconeogenesis, has also been found in the liver of aged offspring born to NR ewes (George et al., 2012).

For skeletal muscle, a reduction of myofiber number (Quigley et al., 2005) , hypertrophy (Zhu et al., 2004) and muscle mass (Fahey et al., 2005) in addition to metabolic alterations regarding oxidative capacity (Zhu et al., 2006), and glucose metabolism (Costello et al., 2008) have been observed in response to maternal NR. Skeletal muscle represents around 40% of total body mass in an adult organism (Janssen et al., 2000), and around 80% of insulin-mediated glucose uptake occurs in this tissue (Ferrannini et al., 1985; DeFronzo and Tripathy, 2009). Thus, alterations in muscle development or metabolism can have profound impacts in the metabolic dysregulation typically seen in SGA offspring. Therefore, studying the effect of prenatal nutrient scarcity on skeletal muscle development, growth, and metabolism is essential to understand its potential impacts on postnatal metabolism and health.

5. Overview of Skeletal Muscle Development and Growth

5.1 Myogenesis

Skeletal muscle development and growth during the fetal stage are accomplished by both, cellular hyperplasia and fusion to originate myofibers (myogenesis), and hypertrophy, which continues postnatally. Myogenesis can be divided into primary and secondary myogenesis.

Primary myogenesis represents the first wave of myofiber formation, accounts for a small percentage of myofibers in the adult and occurs in early pregnancy during the embryonic stage. Specifically, in the sheep, it starts around GD 35 (Maltin, 2008). During primary myogenesis, skeletal muscle precursors called myoblasts fuse to form a primary myotube which will then become a primary myofiber.

Secondary myogenesis starts around GD 62 and accounts for the majority of myofiber formation (Maltin, 2008). During this process, myoblasts fuse to form secondary myotubes around the primary myotubes, which serve as a template, then secondary myotubes become myofibers. Before their fusion to form myotubes, myoblasts can proliferate. Myoblast hyperplasia is essential to provide myogenic precursors, and this process is stimulated by the activation of pathways that typically induce cell proliferation, such as mitogen-activated protein kinase 1 (MAPK1) (aka ERK) (Jones et al., 2001).

During embryonic and fetal development, there are common mesenchymal stem cell precursors from which skeletal muscle develops. Commitment to the myogenic lineage is dependent upon expression of regulatory factors in these common

mesenchymal stem cells precursors, which is in part controlled by the Wingless-type MMTV integration site family (WNT) pathway. An increase in WNT signaling will activate catenin beta 1 (CTNNB1) (aka beta-catenin) to induce expression of paired box 3 (Pax3) and paired box 7 (Pax7), which are markers of differentiation towards a myogenic cell lineage. As a result, there will be an upregulation of myogenic regulatory factors (MRFs) (Cossu and Borello, 1999). MRFs include myogenic differentiation 1 (MYOD1) and myogenic factor 5 (MYF5), which promote the conversion of precursor cells into myoblasts; myogenin, which is involved in the fusion of myoblasts to form myotubes; and myogenic factor 6 (MYF6) (aka MRF4), which is involved in the maturation of myotubes to form myofibers (Figure 1.2) (Stewart and Rittweger, 2006).

Developmental timing is essential in the context of organ susceptibility for programming. In the case of skeletal muscle, because the active formation of new myofibers occurs during early to mid-gestation, it is expected that environmental insults applied during this stage would primarily impair the number of formed myofibers. This effect cannot be reversed postnatally because myofiber formation is not an active process after birth in livestock species (Figure 1.3) (Wigmore and Stickland, 1983).

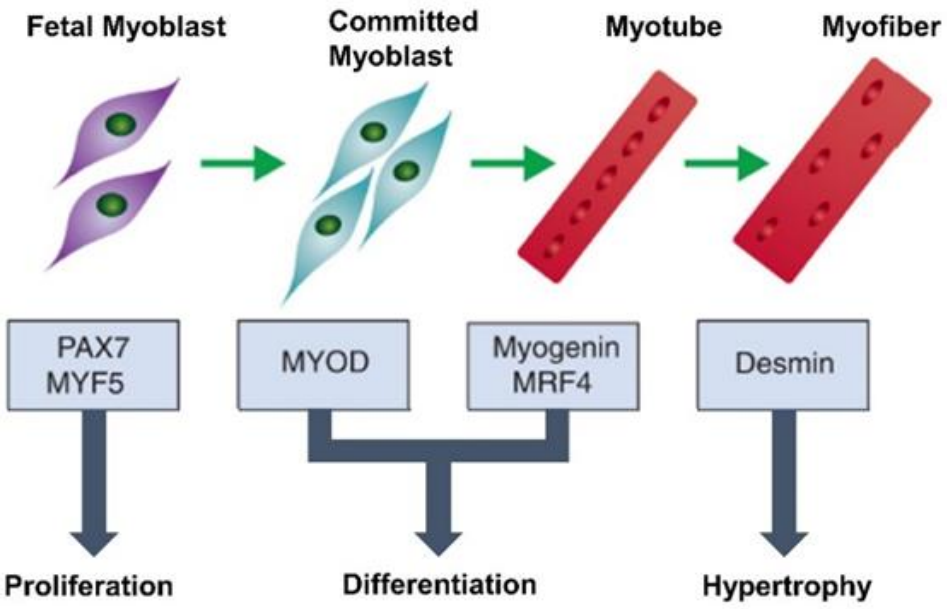

Figure 1.2 Schematic Representation of Skeletal Muscle Development. During the initial stages of myogenesis, myoblast proliferation provides precursors for myofiber formation (PAX 7/MYF5 positive cells). Committed myoblasts (MYOD1 positive cells) stop proliferation and begin differentiation. Expression of myogenin promotes myoblast fusion for myotube formation, and MYF6 (aka MRF4) promote myotube maturation to form a myofiber. After maturation, myofibers begin hypertrophy which continues postnatally. Used with permission from Brown (2014).

After myofiber number is fixed, fetal muscle growth continues through hypertrophy, which starts around the second half of pregnancy and remains as an active process postnatally. The study of Wei et al. (2014) found that myofiber area begins to increase around GD 85, which was caused by the addition of myonuclei between GD 85 and 100. The same study found that myoblast proliferation was completed by GD 100, and followed by an increase in myofiber size likely due to intracellular protein deposition. Skeletal muscle hypertrophy through protein deposition is a very active process in the neonate and depends on the balance between rates of protein synthesis and degradation. Hypertrophy continues during postnatal life. Thus, alterations in myofiber size as a consequence of a prenatal stimuli have the potential to be corrected postnatally (Figure 1.3).

5.2 Pathways for Skeletal Muscle Hypertrophy and Atrophy

Protein deposition is essential for muscle growth and hypertrophy (Yao et al., 2008) and is dependent upon an increase in the net balance of protein synthesis and protein degradation (Brown, 2014). Several molecular pathways interact to control protein synthesis and degradation in skeletal muscle, and most of them respond to nutrient availability and abundance of anabolic signals.

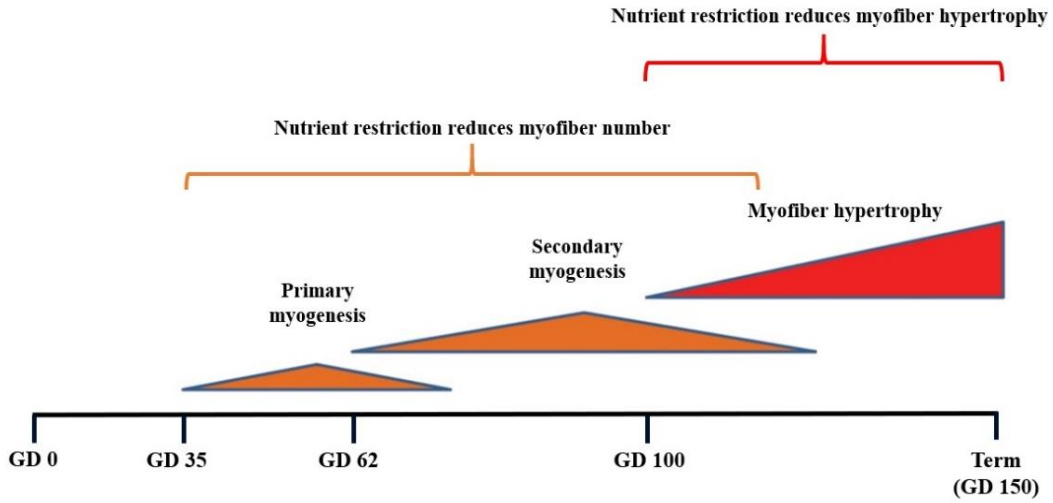

Figure 1.3 Developmental Window of Sensitivity for Skeletal Muscle. The concept, window of sensitivity, refers to the time during gestation in which an organ is susceptible to influence from external stimuli. In skeletal muscle, stages of active myofiber formation (primary and secondary myogenesis) occur primarily during early and mid-gestation. If an insult such as nutrient restriction is applied during this time, it is expected to impair myofiber formation. In contrast, if nutrient restriction occurs in late gestation, it is more likely to affect myofiber hypertrophy and not number, because the peak time for myofiber formation has already passed. GD (Gestational day). Used with permission from Du et al. (2010).

IGF1 - Insulin/PI3K/AKT1 Pathway

Insulin like growth factor 1 (IGF1) is an anabolic factor, which is particularly relevant to promote the growth of placental and fetal tissues, including skeletal muscle (Fowden, 2003). IGF1 signaling is initiated by binding to the IGF1 receptor (IGF1R), which leads to the phosphorylation of insulin receptor substrate 1 (IRS1) (Egerman and Glass, 2014). Insulin acts as an anabolic hormone in addition to its role in glucose metabolism. Binding of insulin to insulin receptor (INSR) leads to phosphorylation of IRS1, and activation of downstream targets that are common for both, IGF1 and insulin signaling. Both insulin and IGF1 can bind to either IGF1R or INSR to activate this signaling pathway, but with greater affinity for their respective receptor (Belfiore et al., 2009).

Activation of IRS1 will subsequently induce activation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) (aka PI3K) which will finally phosphorylate and activate AKT serine/threonine kinase 1 (AKT1) (aka AKT). Activation of AKT1 is one of the regulatory factors for MTOR complex 1 (aka mTORC1) which induces protein translation and hence, has a profound impact in skeletal muscle hypertrophy (Rommel et al., 2001). Additionally, active AKT1 blocks the activity of forkhead box (FOXO) transcription factors. This leads to reduced expression of ubiquitin ligases, decreasing protein degradation in skeletal muscle. As a result of a stimulatory effect on protein synthesis, and inhibitory effect on protein degradation, the activation of AKT1 leads to increased protein deposition in skeletal muscle (Bodine et al., 2001).

MTOR Pathway

Mechanistic target of rapamycin (MTOR) signaling pathway includes both, MTOR complex 1 (previously known as mTORC1) and MTOR complex 2. MTOR complex 1 is involved in the regulation of protein synthesis and deposition to promote skeletal muscle hypertrophy. MTOR complex 2 is primarily involved in the promotion of cell proliferation (Wu, 2013a). MTOR complex 1 is associated to regulatory associated protein of MTOR complex 1 (RPTOR) (previously known as RAPTOR) (Kim et al., 2002) and is activated by phosphorylation of its serine 2448 residue, whose stimulation depends on the energy status of the cell and its nutrient availability. Insulin, IGF1, AKT1, and amino acids such as leucine and arginine have a stimulatory effect on MTOR complex 1 activity (Yoon, 2017). Particularly, the effect of amino acids has been related to translocation of MTOR complex 1 to the lysosome, where it comes in close proximity to the activator protein Ras homolog, mTORC1 binding (RHEB) (Saxton and Sabatini, 2017). In contrast, the activity of MTOR complex 1 is inhibited by glucocorticoids (Shimizu et al., 2011), protein kinase AMP-activated catalytic subunit alpha 2AMP-dependent kinase (PRKAA2) (aka AMPK), and myostatin (Rodriguez et al., 2014).

When active, MTOR complex 1 phosphorylates two proteins, ribosomal protein S6 kinase B1 (RPS6KB1) (aka S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) which is a translational repressor factor. Phosphorylation of RPS6KB1 leads to activation of ribosomal protein S6 (RPS6), which facilitates RNA

translation and protein synthesis. Phosphorylation of EIF4EBP1 leads to the inactivation of this protein, thus preventing its translational repressor effect (Wu, 2013a).

Myostatin Pathway

Myostatin, a member of the transforming growth factor beta (TGFB) family, is a primary negative regulator of muscle hypertrophy (Rodriguez et al., 2014). This signaling pathway is mediated by binding of myostatin to serine/threonine receptors that activate SMAD family member 2 (SMAD2) and SMAD family member 3 (SMAD3) by phosphorylation. SMAD 2,3 form a complex with SMAD family member 4 (SMAD4). The complex is then translocated to the nucleus and controls the transcription of target genes involved in the inhibition of muscle hypertrophy (Trendelenburg et al., 2009). Myostatin also inhibits the expression and phosphorylation of AKT1, which is involved in the activation of MTOR complex 1 pathway for protein deposition (Morissette et al., 2009).

Other authors suggest that myostatin plays a role to inhibit myoblast proliferation, having the potential to affect myofiber number by decreasing hyperplasia during skeletal muscle development (Joulia et al., 2003). It has also been suggested that myostatin can upregulate E3 ubiquitin ligases to induce protein degradation (Lokireddy et al., 2011), but this effect is less well established (Egerman and Glass, 2014).

Glucocorticoid-Mediated Atrophy Pathways

Protein deposition depends on both protein synthesis and degradation. Thus, the net effect on myofiber hypertrophy depends on the balance between those rates.

Decreased fetal muscle weight has been associated with lower protein synthesis and downregulation in the MTOR pathway (Zhu et al., 2004). However, makers for protein degradation have been found to be upregulated in skeletal muscle of chronically hypoglycemic fetuses, without alterations in pathways for protein synthesis (Brown et al., 2014). Hence it has been suggested that a decrease in muscle growth at the fetal stage may be the result of upregulation in pathways for muscle atrophy (Brown, 2014).

Glucocorticoids are involved in the regulation of energy homeostasis and play a role in controlling protein, carbohydrate, and fatty acid metabolism in skeletal muscle (Yoon et al., 2017). Different conditions increase the level of circulating glucocorticoids, such as severe deficit of insulin, sepsis, and starvation (Braun and Marks, 2015). Overall, these conditions represent a status of stress and nutrient deficiency in the organism in which catabolic processes are predominant. Accordingly, glucocorticoids have been recognized as activators of muscle atrophy (Menconi et al., 2007).

The study of Shimizu et al. (2011) found that binding of glucocorticoids to glucocorticoid receptor (NR3C1) inhibits the activity of MTOR complex 1 and stimulates muscle atrophy by upregulating expression of the starvation-sensitive transcription factor Kruppel like factor 15 (KLF15). KLF15 was found to upregulate expression of the mitochondrial isoform of the enzyme branched-chain amino acid transaminase 2 (BCAT2). This enzyme mediates the first step in branched-chain amino acid catabolism in skeletal muscle, which would decrease the availability of leucine, a branched-chain amino acid that has a stimulatory effect on MTOR complex 1 activity (Figure 1.4).

Upregulation of KLF15 is also involved in the induction of a metabolic shift from glucose to fatty acid oxidation in skeletal muscle and muscle-liver cross-talk to support gluconeogenesis. These effects will be discussed later in this chapter.

Upregulation of KLF15, in response to glucocorticoids, was also shown to increase the expression of the ubiquitin ligases F-box protein 32 (FBXO32) (aka Atrogin 1) and tripartite motif containing 63 (TRIM63) (aka MURF-1) (Shimizu et al., 2011). Other authors have also observed an upregulation of these ubiquitin ligases in response to glucocorticoids (Wray et al., 2003). FBXO32 and TRIM63 are involved in the proteasomal-mediated protein degradation system, where the majority of muscle proteins are degraded (Sandri, 2013). Inhibition of MTOR complex 1 activity has also been associated with upregulation of FBXO32 and TRIM63, which is indicative of both, reduced protein synthesis and increased protein degradation (Schakman et al., 2013).

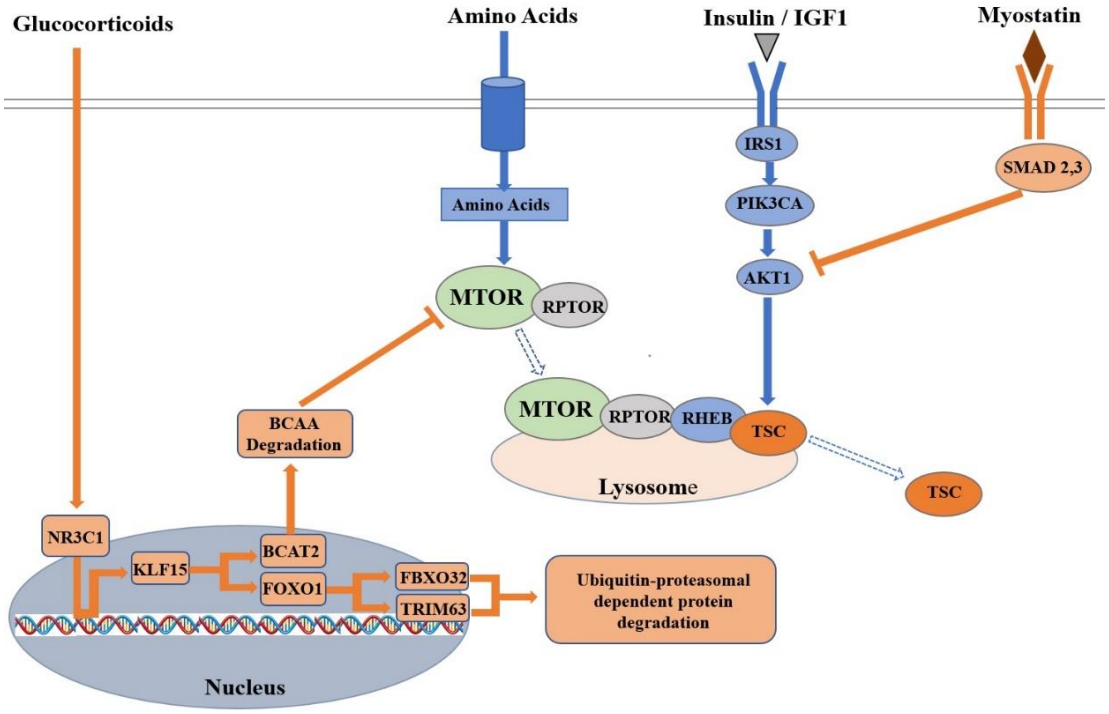

Figure 1.4 Major Regulatory Pathways in Skeletal Muscle Protein Deposition. Protein deposition depends on the rate of protein synthesis and degradation. Pathways that stimulate protein synthesis in skeletal muscle are shown in blue. Amino acids (primarily the branched-chain amino acid leucine, and arginine) induce translocation of MTOR complex 1 to the lysosome, where the complex is activated by RHEB. TSC has inhibitory activity over RHEB. Insulin and IGF1 activate AKT1, which activates RHEB by inducing its separation from the inhibitory factor TSC. Pathways that inhibit protein synthesis or stimulate protein degradation are shown in orange. Myostatin inhibits MTOR through inactivation of AKT1. Glucocorticoids bind to their receptor (NR3C1) to induce expression of KLF15. It is suggested that this decreases activation of MTOR through increased breakdown of branched-chain amino acids (BCAA) via BCAT2. KFL15 upregulation would also increase protein degradation through upregulation of the ubiquitin ligases FBXO32 and TRIM63. PRKAA2 (aka AMPK) also has an inhibitory effect on MTOR when AMP is increased in the cell (not shown) (Based on data from Shimizu et al., 2011; Egerman and Glass, 2014; Yoon, 2017).

Role of Apoptosis in Skeletal Muscle Atrophy

Apoptosis refers to programmed cell death, and while it is typically associated with disease stages, it plays an active role in healthy tissues undergoing fast proliferation or remodeling (Dupont-Versteegden, 2006) and is essential for normal development during the embryonic and fetal stage (Brill et al., 1999).

Different molecular pathways can trigger apoptosis, but in skeletal muscle, the BCL2 apoptosis regulator (BCL2) - BCL2 associated X (BAX) system has been found to be a primary pathway involved in this process (Sandri and Carraro, 1999). The BCL2 family includes anti-apoptotic proteins, such as BCL2 itself, and proapoptotic factors such as BAX. The transcription factor tumor protein 53 (TP53) (aka p53) has proapoptotic action through induction of BAX expression without upregulation of the anti-apoptotic proteins of BCL2 family (Nicholson, 1999). Interestingly, results from mouse models suggest that in addition to its role in apoptosis, BCL2 is also required for normal muscle development (Dominov et al., 2001) which may lead to difficulty in interpretation of findings when measured at the fetal stage.

Most of the research in skeletal muscle has focused on apoptosis in postnatal disease models, creating a gap in knowledge regarding the role of apoptosis in normal skeletal muscle development, and in response to different stimuli that may alter the intrauterine environment (Sandri et al., 2001). However, indicators of increased apoptosis in response to nutrient restriction have already been reported in placenta (Belkacemi et al., 2009), fetal ovaries (Lea et al., 2006), and fetal kidney (Pham et al., 2003; Tafti et al., 2011).

For skeletal muscle, one of the few studies conducted in fetal tissue found at least two waves of apoptosis during myogenesis in human muscle. One of them occurred in early myogenesis with the apparent purpose of removing excess cells to control the number of myofibers formed. The second wave occurred by mid-pregnancy, and its purpose appeared to be the acquisition of final myofiber size and shape (Fidziañska, 1996). The study of Fidziañska (1996) did not provide information regarding apoptosis in fetal skeletal muscles in later stages of the pregnancy. Thus, the potential existence of additional waves of apoptosis remains unknown.

Several authors have described upregulation of apoptosis in postnatal skeletal muscle atrophy (Allen et al., 1999; Whitman et al., 2005). This is supported by the myonuclear domain hypothesis, which suggests that each myonucleus is able to support a specific amount of cytoplasm. Addition of nuclei during muscle development, or regeneration, allow for an increase in myofiber cytoplasm (Rosenblatt et al., 1994), while in atrophy, a loss of myonuclei reduces the cytoplasm that can be supported in certain myofiber. Apoptosis has been suggested as the mechanism responsible for the exclusion of nuclei during atrophy. This would lead to decreased cytoplasm, and myofiber size, without the death of the cell (Hikida et al., 1997) because apoptotic and normal myonuclei have been observed to coexist within the same myofiber.

5.3 Myofiber Type Composition

Skeletal muscle is composed of a mosaic of myofiber types with different metabolic characteristics. The most general classification of myofibers divides them into type I myofibers (or slow-twitch) and type II (fast-twitch). Myofibers formed

during primary myogenesis are primarily type I, while type II myofibers are mainly formed during secondary myogenesis (Maltin, 2008). Myosin heavy chain (MYH) is specific for type I and type II myofibers. However, there is plasticity in regard to myofiber type as interconversion between type I and IIA myofibers, and between type IIA and IIB myofibers have been described (Picard, 2002; Maltin, 2008) (Figure 1.5). During muscle development, embryonic and fetal isoforms of MYH are expressed and later replaced by adult isoforms near term or postnatally. In altricial species, the expression of the adult MYH isoform occurs in late gestation, specifically around GD140 in the sheep (Finkelstein et al., 1992).

Type I myofibers have a high mitochondrial dotation, elevated oxidative capacity and high insulin sensitivity, while type II myofibers have fewer mitochondria, lower oxidative capacity, low insulin sensitivity, and are primarily glycolytic (He et al., 2001). Within type II myofibers, type IIA and IIB are the major subtypes, and the most abundant in sheep longissimus dorsi muscle (Peinado et al., 2004). Type IIA myofibers are oxidative-glycolytic, which means that they show intermediate oxidative capacity, while type IIB are strongly glycolytic. Given these differences among myofiber types, the proportion of type I versus type II myofibers is an important factor that determines the metabolic characteristics in skeletal muscle.

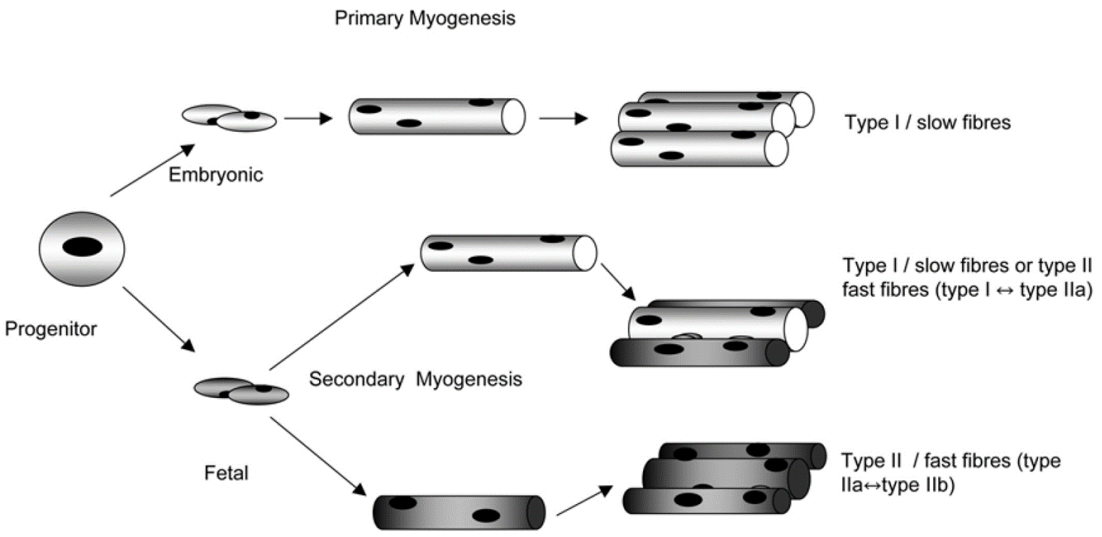

Figure 1.5 Myofiber Type Determination During Myogenesis. Primary myogenesis accounts for a fewer number of myofibers than secondary myogenesis, and those are primarily type I myofibers. Type I and type II myofibers are produced in secondary myogenesis. There is an interconversion potential between the myofibers that are closer in metabolic characteristics (Type I to type IIa, and type IIa to type IIb and vice versa). Plasticity of myofibers for interconversion is maintained postnatally. Reprinted with permission from Maltin (2008).

6. Overview of Skeletal Muscle Impact in Whole-Body Metabolism

Skeletal muscle plays an essential role in locomotion and structural support, but it is also involved in several functions that regulate energy metabolism. Examples of these functions are the capacity for oxidation of fatty acids, glucose, and some amino acids, storage of glycogen (Argilés et al., 2016), and support of gluconeogenesis in liver and kidney through the release of the amino acids, alanine and glutamine (Marliss et al., 1971; Garber et al., 1976) which are synthesized in muscle using BCAA and α-ketoglutarate as precursors (Wu, 2013b). Skeletal muscle is essential in the regulation of glucose metabolism because about 80% of insulin-induced glucose uptake occurs in this tissue (Ferrannini et al., 1985; DeFronzo and Tripathy, 2009). Because skeletal muscle represents 40% of body mass in adult organisms (Janssen et al., 2000), any alteration in muscle mass or metabolism will significantly impact whole-body metabolism (Brown, 2014). As an example, it has been shown that insulin resistance at the skeletal muscle level is one of the primary metabolic alterations leading to type 2 diabetes in humans (DeFronzo and Tripathy, 2009).

Solute carrier family 2 member 4 (SLC2A4) (aka GLUT4) is the major glucose transporter in skeletal muscle, and its action is insulin dependent. The abundance and activity of this transporter are essential for insulin-mediated glucose uptake (Scheepers et al., 2004). SLC2A4 proteins are stored in cytoplasmic vesicles and translocated to the plasma membrane in response to insulin stimulation. Once in the membrane, glucose is transported into the cell through SLC2A4 by facilitative transport.

Translocation of SLC2A4 is induced by activation of the PI3K/AKT1 pathway after binding of insulin to its receptor (INSR) (Kohn et al., 1996). IGF1 can also trigger the activation of this pathway by binding to its receptor or to INSR (Mora et al., 1995; Alessi et al., 1996; Belfiore et al., 2009). This leads to phosphorylation of IRS1, and activation of PIK3CA (aka PI3K) which leads to activation of AKT1. Once active, AKT1 phosphorylates TBC1 domain family member 4 (TBC1D4) (aka AS160), to induce the translocation of SLC2A4 from the cytoplasm to the sarcolemma (Figure 1.6) (Taniguchi et al., 2006). Muscle contraction can also stimulate SLC2A4 translocation, which becomes relevant during postnatal life (Gao et al., 1994).

Upregulation of SLC2A4 in skeletal muscle begins late in fetal life and continues postnatally when this glucose transporter reaches maximum functionality (Stuart et al., 2000). Solute carrier family 2 member 1 (SLC2A1) (aka GLUT1) is a ubiquitous glucose transporter whose action is not dependent on insulin, but directly in response to glucose concentration instead. This glucose transporter plays a major role during prenatal life (Zorzano et al., 2005).

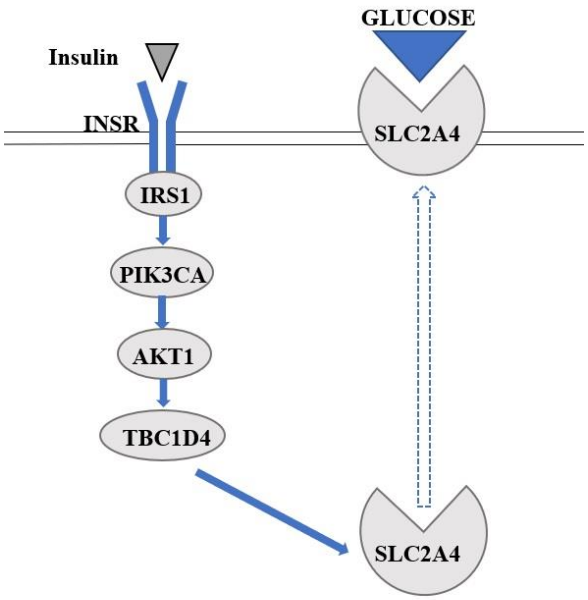

Figure 1.6 Insulin-mediated SLC2A4 Translocation. Around 80% of insulin-mediated glucose uptake occurs in skeletal muscle. Insulin binds to its receptor (INSR) to activate the downstream target AKT1, which will activate TBC1D4 to induce SLC2A4 translocation from cytoplasmic vesicles to the sarcolemma. Because of a high level of homology, IGF1 can also activate this pathway through its receptor (IGF1R) or binding to INSR (not shown). Used with permission from Morrison et al. (2010).

Another factor that influences the metabolic characteristics of skeletal muscle is myofiber type composition, which impacts glucose metabolism and fatty acid oxidation. Type I myofibers are primarily oxidative and more sensitive to insulin than type II myofibers. Thus, its proportion shows a positive correlation with fatty acids and glucose oxidation, and with insulin-mediated glucose transport and whole-body insulin sensitivity (Lillioja et al., 1987; Fisher et al., 2017).

The ability of skeletal muscle to oxidize fatty acids provides metabolic plasticity under conditions such as starvation or exercise, which spares glucose for tissues that depend primarily on this energy source. KLF15, a transcription factor expressed primarily in skeletal muscle and adipose tissue, but also in liver, acts as a starvation sensor and has a major role regulating whole-body metabolism (Gray et al., 2007; Haldar et al., 2012). One of its effects is to induce a metabolic switch from glucose oxidation to fatty acid oxidation in response to fasting (Takeuchi et al., 2016). A suggested mechanism mediating this role is the upregulation of mitochondrial acetyl-CoA synthetase, which synthesizes acetyl-CoA for mitochondrial oxidation (Yamamoto et al., 2004).

It has also been described that KLF15 upregulates hepatic gluconeogenesis under starvation conditions. A suggested mechanism for this function is an increase in BCAT2 transcription in skeletal muscle (Gray et al., 2007). BCAT2 is a mitochondrial enzyme involved in branched-chain amino acid (BCAA) transamination in muscle. Skeletal muscle can synthesize alanine from BCAA and release it into the circulation to be taken up by the liver and used as a gluconeogenic substrate (Wu, 2013b). This crosstalk

between muscle and liver is relevant to maintain euglycemia under starvation conditions, and the regulatory role of KLF15 in this process has been described during postnatal life (Fan et al., 2018). However, to our knowledge, it has not been determined if the starvation sensor function of KLF15 is already active during fetal development.

7. Effect of Maternal Nutrient Restriction in Fetal Skeletal Muscle Growth and Metabolism

During fetal development, tissues have different priorities regarding nutrient partitioning, so that under severe maternal nutrient restriction, growth and development of vital organs such as the brain and heart will be prioritized over non-vital tissues, such as skeletal muscle (Desai et al., 1996). As a consequence, fetal skeletal muscle development is highly susceptible to nutritional insults.

Because of the developmental timing of fetal skeletal muscle, myogenesis is expected to be altered in response to nutritional insults during early to mid-gestation, while alterations in myofiber hypertrophy are primarily expected from mid to late gestation. A peri-conception treatment of 50% NR applied from 18 days before ovulation to 6 days after ovulation found a tendency for decreased myofiber number in sheep semitendinosus muscle at GD 75 (Quigley et al., 2005). Muscle weight and myofiber cross-sectional area were not affected in this study. This was expected given that the NR insult was not applied during the window of sensitivity for myofiber hypertrophy.

A sheep model of 50% NR between GD 28 and 78 found a decrease in the number of secondary myofibers in longissimus dorsi at GD 78. This was suggested to be

associated with impaired myoblast proliferation during secondary myogenesis (Zhu et al., 2004), a process that begins around GD 62 in the sheep (Maltin, 2008). The study of Zhu et al. (2004) also found a decrease in myofiber area in longissimus dorsi of fetuses from NR dams. This finding was associated with a reduction in MTOR and RPS6KB1 protein phosphorylation without upregulation in ubiquitin-labeled proteins. These results were indicative of decreased protein synthesis in the absence of evidence for increased protein degradation.

In a later study from the same group, lambs were naturally delivered after 50% NR between GD 28 and 78 and fed ad libitum until eight months of age. At this time, longissimus dorsi was collected, and no differences in myofiber hypertrophy or muscle weight were found (Zhu et al., 2006). Another model of 50% NR from GD 85 to 115 found a decrease in weight of longissimus dorsi in 14-day-old lambs (Fahey et al., 2005). Collectively these findings support the fact that nutritional alterations in early prenatal life affect myogenesis, while nutritional restriction during mid or late pregnancy primarily affects myofiber hypertrophy. This last effect can be partially compensated after birth because muscle hypertrophy continues during postnatal life, and neonates experience accelerated rates of protein deposition when nutrients are available (Rudar et al., 2019).

Decreased fetal growth after prenatal nutrient restriction is usually followed by compensatory growth during postnatal life (De Blasio et al., 2007) However, in the long term, this compensatory growth will favor adipose tissue deposition instead of muscle growth. For example, 280-day-old lambs born to dams that were subjected to 50% NR

from GD 28 to 78 were heavier than controls, had increased renal and pelvic adipose tissue, and a tendency for decreased weight in longissimus dorsi and semitendinosus muscles. This study also found evidence of hyperglycemia and altered insulin secretion after a glucose tolerance test (Ford et al., 2007). Accordingly, 1-year-old offspring born to sheep under 50% NR from GD 110 to term, showed evidence of glucose intolerance as indicated by increased areas under the curve for glucose and insulin (Gardner et al., 2005).

Indicators of alterations in glucose and insulin metabolism have been found in skeletal muscle at the fetal stage after maternal nutrient restriction. A sheep model of 50% NR from GD 104 to 127 produced upregulation of *SLC2A4*, *INSR*, and *IGF1* mRNA in fetal triceps brachii muscle at GD127 (Costello et al., 2008). These results suggest a metabolic programming effect that could be partly responsible for the compensatory growth that SGA animals experience early in postnatal life. The authors also suggested that an initial upregulation in insulin receptor may play a role in the development of metabolic diseases later in postnatal life (Costello et al., 2008). Supporting data was provided by a study that induced 70% NR from GD 0 to 6 in sheep. This treatment was found to upregulate *IRS1* and *SLC2A4* mRNA expression in quadriceps muscle at GD 137±1 (Lie et al., 2014).

The study of Costello et al. (2008) also measured myofiber and capillary density and compared triceps brachii and soleus muscles for this analysis. Decreased myofiber and capillary density were found in the triceps brachii, but not in the soleus. A possible explanation is the myofiber type composition in these two muscles, since triceps brachii

has a higher proportion of type II myofibers and the soleus is primarily type I. Most type I myofibers are formed in early gestation (primary myogenesis), so they may remain unaltered when the NR treatment is applied outside the window of sensitivity. Soleus muscle has also been identified by other investigators as relatively resistant to maternal nutrient restriction (Ward and Stickland, 1991). These results indicate that regardless of having been exposed to the same treatment, a muscle-specific response can occur.

Maternal nutrient restriction has also been shown to alter myofiber type composition in skeletal muscle. A 50% NR from GD 28 to GD 78 has been associated with increased content of type IIb myofibers in longissimus dorsi of 8-month-old lambs (Zhu et al., 2006). This study also found decreased activity of the enzyme carnitine palmitoyltransferase-1, which is involved in fatty acid oxidation, and accordingly, intramuscular triglyceride (IMTG) content was increased. These findings suggest metabolic programming in skeletal muscle which would impair oxidative capacity and insulin sensitivity, as type II myofibers are primarily glycolytic and less insulin sensitive than type I myofibers (He et al., 2001). Accumulation of IMTG has also been recognized as a cause for disruption in insulin signaling, and insulin resistance in skeletal muscle (Corcoran et al., 2007).

Contradictory results have been found in longissimus dorsi of 14-day-old lambs in which an increase in type I myofibers was found after 50% NR from GD 30 to 70 (Fahey et al., 2005). The difference in offspring age at which these two studies were conducted may be a cause for these conflicting results. However; the results of Zhu et al.

(2006) are supported by the study of Costello et al. (2008), in which a sheep model of 50% NR from GD 104 to 127 was shown to reduce type I myofiber content in fetal triceps brachii muscle at GD127. Myofiber type composition conserves a certain level of plasticity during postnatal life in response to some stimuli such as exercise. Thus, more research is needed to confirm the long-lasting effect of myofiber type programming during fetal development (Brown, 2014).

The discussed data supports the existence of a programming effect on fetal myofiber formation, skeletal muscle mass, metabolism, and myofiber composition in response to maternal nutrient restriction. Nevertheless, while available studies have typically considered fetuses within NR treatments as a single experimental group, preliminary results from our laboratory have routinely shown an ample variation of fetal weights in response to NR in the sheep. Thus, studies were conducted to evaluate the effect of maternal NR on fetal skeletal muscle growth and metabolism while accounting for differences in fetal growth within NR fetuses in the sheep.

CHAPTER II

EFFECT OF MATERNAL NUTRIENT RESTRICTION ON PATHWAYS FOR FETAL SKELETAL MUSCLE GROWTH IN SGA AND NON-SGA SHEEP FETUSES

1. Introduction

Maternal nutrient restriction (NR) during pregnancy is a cause for small for gestational age (SGA) offspring, which exhibit a greater risk to develop metabolic syndrome in adulthood (Barker and Clark, 1997; Ozzane et al., 1999). Several fetal organs can be affected by maternal NR (Osgerby et al., 2002; Vonnahme et al., 2003; Lloyd et al., 2012; Satterfield et al., 2013), but skeletal muscle is among the most susceptible ones because of nutrient prioritization to vital organs (Desai et al., 1996). SGA offspring have typically been associated with decreased muscle mass caused by reduced myofiber number (Quigley et al., 2005), impaired hypertrophy (Fahey et al., 2005), or both (Zhu et al., 2004).

Skeletal muscle plays an essential role in the regulation of oxidative and glucose metabolism, as it represents about 40% of body mass in healthy individuals (Janssen et al., 2000), and roughly 80% of insulin-mediated glucose uptake occurs in this tissue (Ferrannini et al., 1985). Thus, a decrease in muscle mass leads to an unhealthy phenotype that is itself a predisposition for metabolic alterations (Kensara et al., 2005; Atlantis et al., 2009). Additionally, in an agricultural context, decreased muscle mass in livestock species will affect meat production efficiency, leading to economic loses in

producers, and decreasing the production of protein for human consumption, which further impacts human health (Wu et al., 2006).

During fetal development, myofiber formation (myogenesis) is an active process in early to mid-gestation (Maltin, 2008) and depends on myoblast proliferation and fusion. Muscle growth continues through hypertrophy in late gestation and postnatally, and it accounts for the major increase in muscle mass during late prenatal and postnatal stages (McCoard et al., 2001). Hence, impaired prenatal muscle hypertrophy has the potential to be compensated postnatally, but it has been demonstrated that reductions in muscle mass persist until late postnatal life in low-birth-weight humans (Kensara et al., 2005). This is, in part, explained because SGA offspring typically experience accelerated postnatal growth, but this favors adipose tissue deposition instead of muscle (De Blasio et al., 2007).

Skeletal muscle hypertrophy depends upon protein synthesis and degradation. A major regulator of protein synthesis is mechanistic target of rapamycin (MTOR), which induces protein translation when active. The activity of this pathway relies on nutritional and energy availability and is activated by anabolic signals such as insulin and insulin like growth factor 1 (IGF1), and amino acids such as leucine and arginine (Yoon, 2017). Amino acids also act as building blocks for protein synthesis, so their availability is essential to support this process. MTOR signaling is inhibited by negative regulators of muscle mass such as myostatin and glucocorticoids (Rodriguez et al., 2014; Braun and Marks, 2015). At the fetal stage, MTOR activity has been found to be downregulated in sheep fetal skeletal muscles in response to maternal NR (Zhu et al., 2004). However,

markers for protein degradation have been found to be upregulated in fetal muscles without modifications in pathways for protein synthesis in response to chronic glucose restriction during the last 40% of pregnancy (Brown et al., 2014). Accordingly, we have previously observed no differences in MTOR pathway activity in our sheep model of maternal NR at gestational day (GD) 125 (Keith et al., unpublished results). It has been suggested that upregulation on pathways for protein degradation, which typically mediate atrophy in adult muscles, may be upregulated in fetal muscles under maternal NR conditions and be the cause for decreased protein deposition (Brown and Hay, 2016).

Glucocorticoids are a major negative regulator of protein deposition during muscle atrophy in adult tissues (Braun and Marks, 2015), but their action has also been demonstrated in fetal muscles (Gokulakrishnan et al., 2012). However, the regulation and effects of this pathway in fetal skeletal muscles have been less studied than in adult tissues (Brown et al., 2014), but it is of interest under altered physiologic conditions that would trigger muscle atrophy in adult tissues, such as undernutrition and cachexia. One pathway of glucocorticoid action is through upregulation of the starvation-sensitive factor Kruppel like factor 15 (KLF15). This factor upregulates the ubiquitin ligases F-box protein 32 (FBXO32) and tripartite motif containing 63 (TRIM63), which are mediators of protein degradation, and indirectly inhibits MTOR through an increase in branched-chain amino acid breakdown (Shimizu et al., 2011). Additionally, KLF15 orchestrate metabolic adaptations to protect glucose homeostasis during starvation (Gray et al., 2007; Haldar et al., 2012). To our knowledge, the responsiveness of KLF15 to

maternal NR, and its potential effects at the fetal stage has not been evaluated, but given its starvation-sensitive characteristic, it is feasible that KLF15 is altered by prenatal nutrient scarcity.

The majority of published studies describing decreased muscle weight and myofiber hypertrophy in response to maternal NR have focused on downregulation of pathways for decreased protein synthesis rather than upregulation in protein degradation. Further, studies have typically considered fetuses from NR dams as a single experimental group regardless of their growth rates. However, our studies in a NR sheep model have shown a spectral phenotype of fetal growth rates, possibly due to maternal adaptation to nutrient scarcity in order to support fetal growth. Due to these observations, we sought to characterize mechanisms by which the placenta adapts to nutritional deprivation and the resultant fetal responses. This study aimed to evaluate the effect of differential responses to maternal NR on myofiber hypertrophy, the glucocorticoid-mediated pathway for muscle atrophy, and its interactions with MTOR activation.

2. Materials and Methods

All experimental procedures in this study were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

2.1 Animal Handling and Tissue Collection

Mature Hampshire ewes of similar frame size, parity, and body condition were fed to meet 100% of their nutritional requirements according to recommendations of the National Research Council (NRC) and used as embryo transfer recipients. A protocol for

estrus synchronization and superovulation was administered to donor and recipient ewes. Hampshire donor ewes were artificially inseminated, and on gestational day (GD) 6, a single embryo was transferred from a donor ewe into the recipient uterus. Ultrasound was used as pregnancy diagnostic on GD 28. On GD 28, ewes were housed in individual pens on concrete and maintained in these conditions until GD 135. Ewes were fed once per day. Rations were adjusted once per week based on weekly measurements of body weight. On GD 35, ewes were randomly divided into a control-fed group which received 100% NRC requirements, and a nutrient-restricted (NR) group, which received 50% of total NRC requirements. Composition of the diet has been published previously (Lassala et al., 2010). Necropsies were conducted on GD 135. At this time, blood samples from fetal umbilical vein and fetal heart were collected to obtain fetal plasma. Soleus and gastrocnemius muscles were dissected and weighed, and samples preserved for further investigation.

Fetuses from ewes fed 100% NRC formed the **Control** group (n=12). Fetuses from NR ewes were divided into quartiles based on fetal weight distribution. The highest (**NR(Non-SGA)** group; n=11) and lowest (**NR(SGA)** group; n=11) quartiles were selected for further investigation (Keith et al., unpublished results).

2.2 Immunofluorescence

Immunofluorescence was used to measure myofiber area. One section of 8 µm was obtained per sample using a cryostat at -16^{o}C. A modification of the protocol described by Wang et al. (2014) was used. Briefly, each section was fixed in cold methanol for 10 minutes and blocked using 10% normal goat serum for 1 hour at room

temperature. After that, an anti-laminin antibody (1:300, catalog number L9393, Sigma, St. Louis, MO) was added for overnight incubation at 4°C. Rabbit IgG was substituted for the primary antibody as a negative control. A secondary antibody (1:500, Alexa Fluor 594®, Thermo Fisher Scientific, Waltham, MA) was then added for one hour of incubation at room temperature. Slides were overlaid using Prolong Antifade (Life Technologies, Carlsbad, CA) without DAPI. Eight non-overlapping areas were imaged per each section, using a Nikon Eclipse Ni-E fluorescence microscope and NIS-Elements AR 4.30.02 64-bit Software (Nikon Instruments Inc., Melville, NY). For quantification, the cross-sectional area of 10 randomly selected cells was measured in each of the eight areas per sample and averaged to get a representative value.

2.3 Cortisol Concentration in Fetal Plasma

Cortisol concentration in plasma was measured using RIA (Catalog number CRCT-1000V-100/500, IVD Technologies, Orange County, CA) according to manufacturer recommendations. To calculate total hormone content in the fetal circulation concentrations were multiplied by blood volume, which was estimated at 110ml/kg fetal weight (Brace, 1983; Brace, 1986).

2.4 Amino Acid Concentration and Total in Fetal Plasma

Amino acid concentration in fetal plasma was measured as described by Satterfield et al. (2013). Briefly, fetal plasma samples were deproteinized, and amino acids content was measured using fluorimetric HPLC with precolumn derivatization using o-phthaldialdehyde. Chromatographic peaks were integrated using Millenium-32 Software (Waters, Milford, MA). To calculate total amino acid content in the fetal

circulation, concentrations were multiplied by blood volume, which was estimated at 110ml/kg fetal weight (Brace, 1983; Brace, 1986).

2.5 RNA Extraction and qPCR

Trizol (Gibco-BRL, Bethesda, MD) was used for RNA extractions. RNA quantity was assessed by spectrophotometry, and quality was evaluated based on RNA Integrity Number (RIN) after running the samples on a bioanalyzer. cDNA was made by reverse transcription using SuperScript II kit and oligo-dT primers (Invitrogen, Carlsbad, CA) by reverse PCR in an Eppendorf Mastercycler Nexus Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). Newly synthesized cDNA was used to measure relative gene expression by qPCR using a 7900 HT fast real-time PCR system (Applied Biosystems, Foster City, CA). SYBR Green (Applied Biosystems, Foster City, CA) was added as a detector and signals were integrated using SDS2.4 software. Primers were designed to quantify the expression of glucocorticoid receptor (*NR3C1*), Kruppel like factor 15 (*KLF15*), forkhead box O1 (*FOXO1*), F-box protein 32 (*FBXO32*), tripartite motif containing 63 (*TRIM63*), branched chain amino acid transaminase 2 (*BCAT2*), myostatin (*MSTN*), and lipoprotein lipase (LPL). *EEF1A2* (gastrocnemius) or *SF3A1* (soleus) were used as housekeeping genes (Pérez et al., 2008) (Table 2.1). Relative mRNA expression was quantified using the ΔΔ CT method as described by Satterfield et al. (2008).

Table 2.1 Primers Used for qPCR Analysis [1]

Target	Forward/Reverse Primers (5'3')	Amplicon Length (bp)	Accession No. or Reference
NR3C1	ATGGATGTTCCTCATGGCGTT CAGATCAGGAGCAAAACACAGC	85	NM_001114186.1
KLF15	ACCTTCTCGTCGCTGAAACG GAACACAGGGTTTGCGAGTC	143	XM_027957891.1
FOXO1	AAATGATGACCCCCAGCTCC GGGCCAGAGGCACTTGTAAA	159	XM_027973596.1
FBXO32	AAAGTCCTTGAAGACCAGCAA AAGCACAAAGGCAGGTCTGT	232	Brown et al., 2014
TRIM63	CATGTGCAAGGTGTTCGGAG GATGGTCTGCACACGGTCAT	133	XM_012115257.2
BCAT2	GAGCCCTCCTGTTCGTCATT GCCCGGATGAATGATGGATCT	100	XM_027978581.1
MSTN	AACGTTTGGCTTGGCGTTAC GCCAGCAACAAGCAGCATAA	145	AM992883.1
LPL	TGGAGATGTGGACCAGCTAGT CCGGTAGGCCTTACTTGGATT	100	NM_001009394.1
EEF1A2	CCTTTGCACCCGTGAACATC TCTTCACGTTGAAGCCGACA	105	XM_027976945.1
SF3A1	TCCATCTCAGCCTTCGACCT CTGGTAGTTGCGCTGCTCTT	111	XM_027980265.1

[1]Forward and Reverse Primers were designed using Primer-BLAST Tool from NCBI with the showed accession numbers as sequence input. Primer sets having a citation instead of accession number were obtained from published articles. All primers were tested for specificity using BLAST tool from NCBI and dissociation curve analysis to confirm amplification of a single product. Standard curves were used to optimize the efficiency of the assay for each primer set.

2.6 Protein Extraction and Western Blots

Total protein from soleus and gastrocnemius muscles were extracted by tissue homogenization in lysis buffer using snap-frozen samples. Protein concentration was measured using Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as standard. Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Total and phosphorylated FOXO1 (1:1000 catalog numbers 2880 and 2486), MTOR (1:1000, catalog numbers 2972 and 2971), EIF4EBP1 (1:1000, catalog numbers 9452 and 9455), RPS6 (1:1000, catalog numbers 9202 and 9206), and MAPK3/MAPK1 (aka ERK1/2) (1:1000, catalog numbers 9102 and 9101) were quantified by western blots as previously described (Spencer et al., 1999) using 50 ug of protein. Total and phosphorylated MTOR proteins were transferred overnight at 4°C and 30V, while all the other proteins were transferred at 100V for 1.5 hours in a cold chamber. An IgG-horseradish peroxidase-conjugated antibody (1:20000 of 1mg/ml stock) (KPL, Bethesda, MD) followed by incubation on SuperSignalTM West Dura (Thermo Scientific, Waltham, MA) were used to detect immunoreactive proteins by chemiluminescence. Proteins were quantified using Quantity One® 4.6.1 Software (Biorad Laboratories, Inc., Hercules, CA) with tubulin (1:10000, catalog number Ab8291, Abcam, Cambridge, MA) and GAPDH (1:10000, catalog number 2118, Cell Signaling Technologies, Danvers, MA) as loading controls for gastrocnemius and soleus respectively.

2.7 Statistical Analysis

Data were analyzed using JMP® Pro 14 software (SAS Institute Inc., Cary, NC). One-way ANOVA ($\alpha = 0.05$) analysis was used to compare the means of control (**n=12**), NR(SGA) (**n=11**) and NR(non-SGA) (**n=11**) groups and differences between specific means were evaluated using Tukey's test.

3. Results

3.1 Fetal and Muscle Weights

Fetal weight at GD 135 was not different between the control and NR(Non-SGA) groups, while it was lower ($P<0.0001$) in the NR(SGA) group (Figure 2.1). Fetal muscle weights of both, gastrocnemius and soleus, followed the same pattern as there were no differences between control and NR(Non-SGA) groups, and both groups had a higher mean weight than the NR(SGA) group ($P<0.0001$) (Figure 2.2).

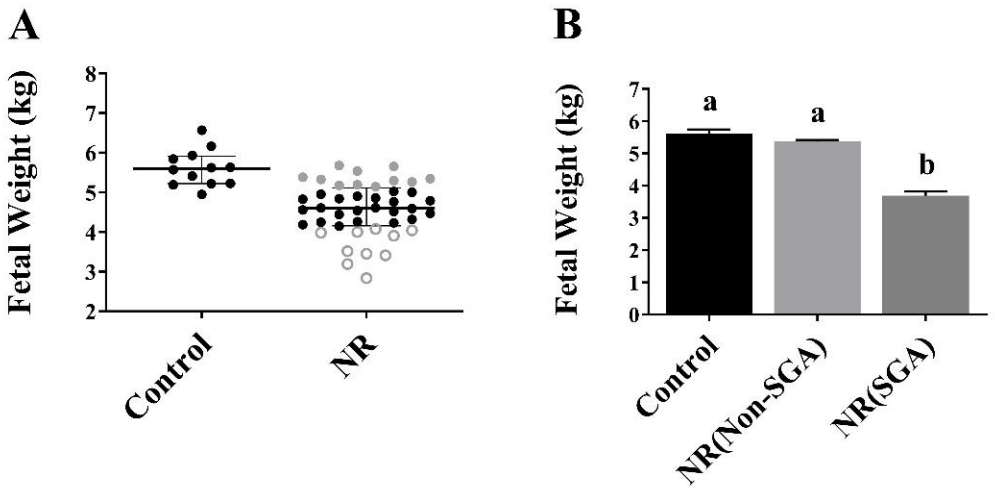

Figure 2.1 Fetal Weights at GD 135 and Experimental Group Formation.
(A) Weight distribution of fetuses from control (n=12) and nutrient restricted (NR) ewes (n=44). Highest quartile (grey closed circles) forms the NR(Non-SGA) group (n=11). Lowest quartile (grey open circles) forms NR(SGA) group (n=11). **(B)** Average fetal weights per group ($\bar{X}$±SEM). Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.0001$).

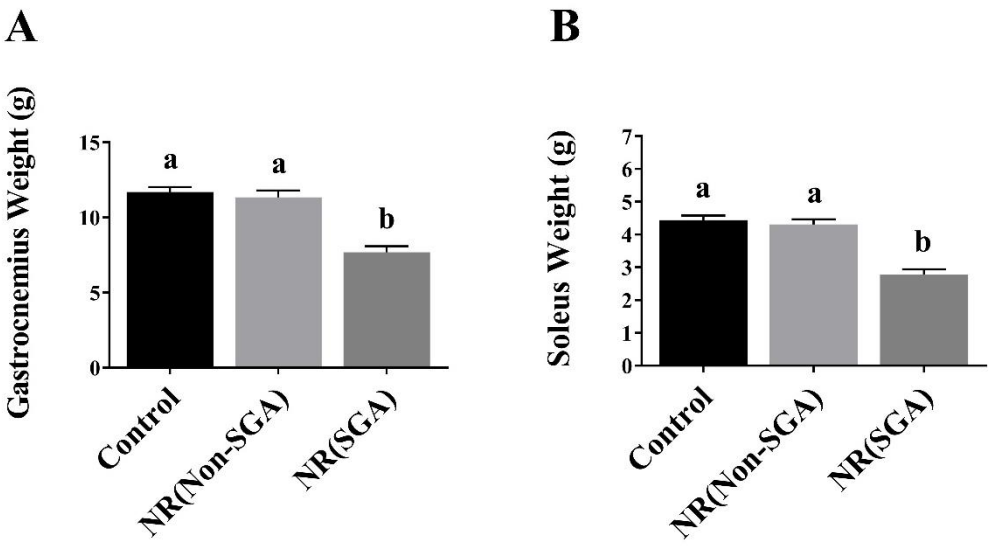

Figure 2.2 Fetal Muscle Weights at GD 135. Mean weight for gastrocnemius **(A)**, and soleus **(B)** per group are shown as $\bar{X}$±SEM. For both muscles, NR(SGA) fetuses had a lower mean weight than NR(Non-SGA) and controls. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.0001$).

3.2 Myofiber Cross-sectional Area

For both, gastrocnemius and soleus muscles, average myofiber cross-sectional area was decreased (P=0.0068 and P=0.0070, respectively) in NR(SGA) group compared to control, while the NR(Non-SGA) group had intermediate values and were not different from either control or NR(SGA) groups (Table 2.2, Figure 2.3A and 2.3B).

Table 2.2 Myofiber Cross-sectional Area per Group in Gastrocnemius and Soleus Muscles at GD 135[1]

Tissue	Control (μm^2)	NR(Non-SGA) (μm^2)	NR(SGA) (μm^2)
Gastrocnemius	1150 ± 71^{a}	991 ± 63^{ab}	838 ± 61^{b}
Soleus	853 ± 55^{a}	719 ± 60^{ab}	586 ± 60^{b}

[1]Means represent measured areas within each muscle for Control, NR(Non-SGA), and NR(SGA) groups. Data is shown as $\bar{X} \pm SEM$. Different superscripts indicate $P<0.01$. Images of myofiber CSA per group in each muscle are shown in Figure 2.3.

3.3 Cortisol Levels in Fetal Plasma

Neither cortisol concentration (µg/dL) (P=0.2026) nor estimated total cortisol content (µg) (P=0.7902) in fetal plasma were different among groups (Figure 2.4).

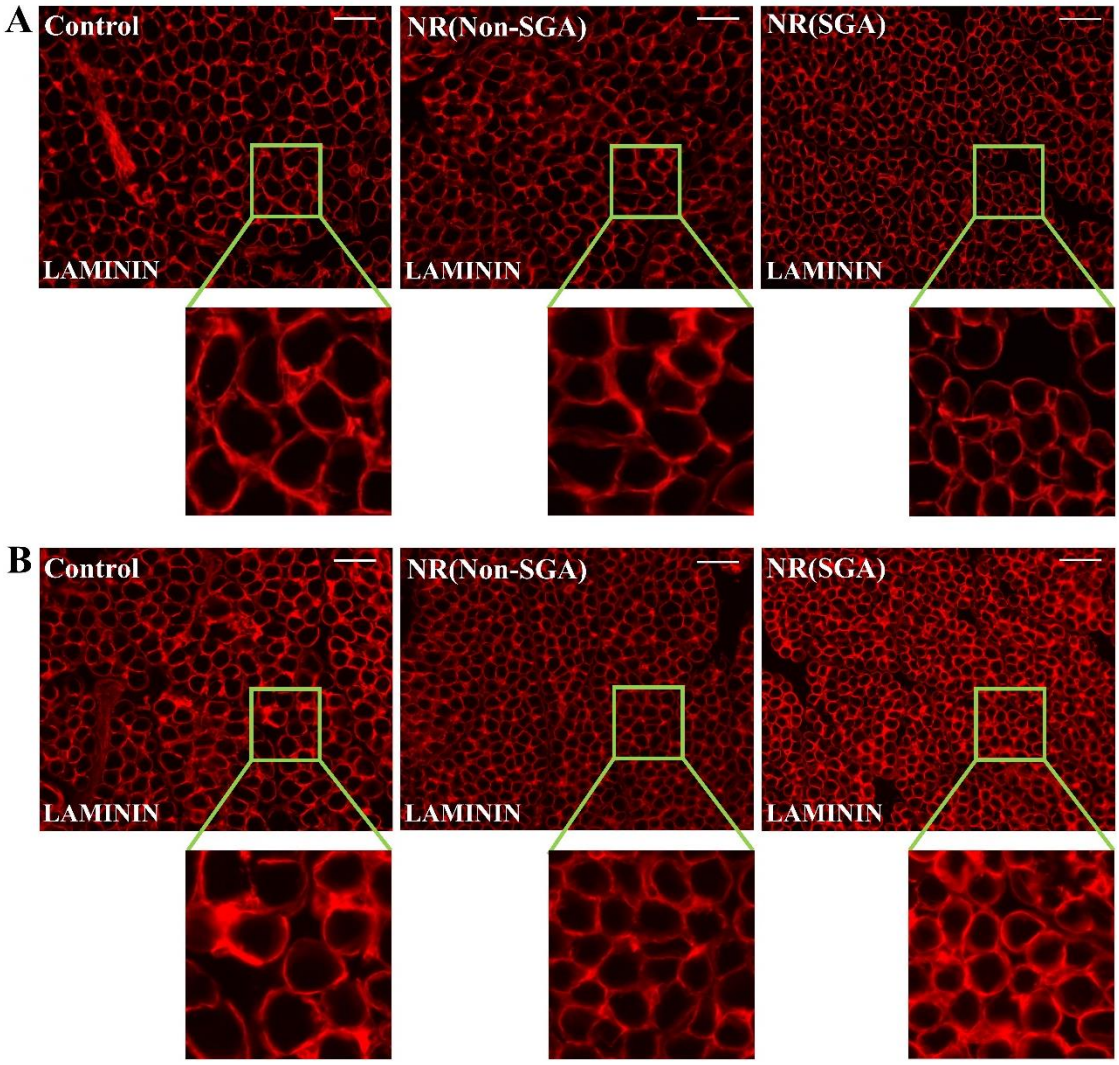

Figure 2.3 Myofiber Cross-Sectional Area in Gastrocnemius and Soleus Muscles at GD 135. Immunoreactive protein for Laminin (Red) are shown for Gastrocnemius **(A)** and Soleus **(B)** in control, NR(Non-SGA), and NR(SGA) groups. Laminin was used as a plasma membrane marker to delineate myofiber cross-sectional area (CSA). Magnified pictures show details of CSA per group, showing smaller areas for myofibers in the NR(SGA) group. CSA quantifications are shown in Table 2.2. For the IgG control (not shown), normal rabbit IgG was substituted for the primary antibody. Pictures were taken at 20X magnification. Scale bar equals 25 µm.

3.4 Amino Acid Levels in Fetal Plasma

Fetal plasma concentration of arginine (Arg), leucine (Leu), and total amino acids were higher ($P<0.05$) in NR(Non-SGA) compared to NR(SGA), while control group had an intermediate concentration and did not differ from the other two groups. Threonine (Thr) and β-alanine concentrations were lower ($P<0.05$) in NR(SGA) compared with control fetuses, while NR(Non-SGA) did not differ from the other two groups. Specific results for the concentration of each amino acid are shown in Table 2.3). The total content in circulation of most individual amino acids, as well as total amino acids, were higher ($P<0.05$) in both, control and NR(Non-SGA) compared to the NR(SGA) group. Specific results for total content of each amino acid are shown in Table 2.4.

3.5 mRNA Expression

Within gastrocnemius muscle, *NR3C1* and *FOXO1* were upregulated in NR(SGA) ($P=0.0099$ and $P=0.0131$, respectively) compared to control, while NR(Non-SGA) did not differ from the other groups. *KLF15* was upregulated in both, NR(Non-SGA) ($P=0.0178$) and NR(SGA) ($P=0.0001$) compared to the control group. *LPL* was upregulated in both, NR(Non-SGA) ($P=0.0289$) and NR(SGA) ($P=0.0330$) compared to control. No differences were found for *FBXO32* ($P=0.3387$), *TRIM63* ($P=0.5647$), *BCAT2* ($P=0.7631$), or *MSTN* ($P=0.3787$) (Figure 2.5). Within soleus muscle, there was an upregulation of *KLF15* in NR(SGA) group ($P=0.0114$) compared to control, while NR(Non-SGA) group did not differ from either control or NR(Non-SGA) groups. *BCAT2* was upregulated in both, NR(Non-SGA) ($P=0.0003$) and NR(SGA) ($P=0.0004$)

groups compared to control. *MSTN* was upregulated in NR(SGA) (0.0259) compared to control. No differences were found for *NR3C1* (P=0.2888), *FOXO1* (P=0.5559), *FBXO32* (P=0.9808), *TRIM63* (P=0.0615), or *LPL* (P=0.4569) (Figure 2.6).

3.6 Protein Expression

For gastrocnemius, there were no differences in the ratio between total and phosphorylated FOXO1 (P=0.7254), MTOR (P=0.7637), RPS6 (P=0.9003), EIF4EBP1 (P=0.7884) and MAPK3/MAPK1 (P=0.4341). Similarly, for soleus, there were no differences in the ratio between total and phosphorylated FOXO1 (P=0.1978), MTOR (P=0.9029), RPS6 (P=0.9896), EIF4EBP1 (P=0.5976), and MAPK3/MAPK1(P=0.7907). For both muscles, there were no differences in either total or phosphorylated content of any of the evaluated proteins (P>0.05) (Figure 2.7 and Figure 2.8).

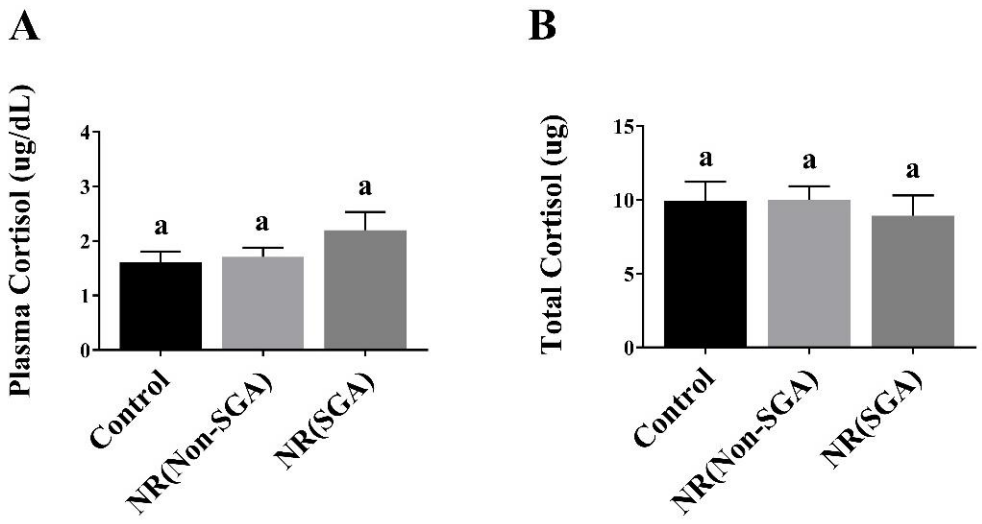

Figure 2.4 Cortisol Levels in Fetal Plasma at GD 135. Concentration and total levels of cortisol in control, NR(Non-SGA) and NR(SGA) are shown in **(A)** and **(B)** respectively. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.05$).

Table 2.3 Amino Acid Concentration in Fetal Plasma at GD 135[1]

Amino Acid	Control (nmol/mL)	NR(Non-SGA) (nmol/mL)	NR(SGA) (nmol/mL)	ANOVA P value
Ala	408.4 ± 29.6	445.4 ± 29.6	329.5 ± 35	0.0661
β-Ala	19.1 ± 1.9 [a]	13.8 ± 1.9 [ab]	6.9 ± 2.2 [b]	0.0031
Arg	289.5 ± 30.9 [ab]	371.9 ± 30.9 [a]	207.7 ± 33.4 [b]	0.0080
Asn	64 ± 4.3 [ab]	69.6 ± 4.3 [a]	50.9 ± 5.1 [b]	0.0427
Asp	5.6 ± 0.9	6.8 ± 0.9	5.3 ± 1	0.5623
Cit	214 ± 19.9	184.8 ± 19.9	147.1 ± 23.3	0.1268
Gln	465.2 ± 60.7	552.1 ± 60.7	328.9 ± 71.9	0.0898
Glu	114.1 ± 18.2	121.2 ± 18.2	117 ± 19.7	0.9633
Gly	351.8 ± 53.9	425.9 ± 53.9	390.5 ± 63.8	0.6323
His	32.5 ± 4.0	36.2 ± 4	29.1 ± 4.7	0.5368
Ile	66.4 ± 6.5	84.1 ± 6.5	66.8 ± 7.4	0.1183
Leu	127.5 ± 8.3 [ab]	157.2 ± 8.3 [a]	123 ± 9 [b]	0.0226
Lys	132 ± 13.2	163.8 ± 13.2	126.5 ± 14.2	0.1374
Met	30.2 ± 3.1	29.8 ± 3.1	27 ± 3.4	0.7655
Orn	193.1 ± 22.1	185.4 ± 22.1	155.9 ± 26.2	0.5463
Phe	86.5 ± 7.1	99.1 ± 7.1	94.6 ± 7.7	0.0556
Ser	597 ± 68.5	686.3 ± 68.5	489.3 ± 74	0.1791
Tau	154.9 ± 30.7	139.1 ± 30.7	142.5 ± 33.1	0.9302
Thr	285.8 ± 34 [a]	246.2 ± 34 [ab]	136.8 ± 40.3 [b]	0.0361
Trp	75.6 ± 3.6	77.7 ± 3.6	76.6 ± 3.9	0.6568
Tyr	74.7 ± 4.6	75.7 ± 4.6	73 ± 5	0.9245
Val	187.3 ± 12.1	213.1 ± 12.1	194.8 ± 13.9	0.3300
Total AA	3976.4 ± 216.7 [ab]	4386.1 ± 216.7 [a]	3429.3 ± 256.4 [b]	0.0375

[1]Data is shown as $\bar{X}$±SEM. Different superscripts indicate significant difference between means.

Table 2.4 Total Content of Amino Acids in Fetal Blood at GD 135[1]

Amino Acid	Control (μmol)	NR(Non-SGA) (μmol)	NR(SGA) (μmol)	ANOVA P value
Ala	250.7 ± 16.8 [a]	267.1 ± 16.8 [a]	138.8 ± 19.8 [b]	0.0003
B-Ala	11.8 ± 1.1 [a]	8.3 ± 1.1 [a]	2.84 ± 1.4 [b]	0.0031
Arg	179.1 ± 18.7 [a]	224 ± 18.7 [a]	87.6 ± 20.2 [b]	0.0005
Asn	39.6 ± 2.8 [a]	41.8 ± 2.8 [a]	21.3 ± 3.3 [b]	0.0026
Asp	3.5 ± 0.5	4 ± 0.5	2.2 ± 0.6	0.1353
Cit	132.7 ± 11.8 [a]	111 ± 11.8 [a]	59.8 ± 14 [b]	0.0038
Gln	289.6 ± 36.1 [a]	333.1 ± 36.1 [a]	135.2 ± 42 [b]	0.0080
Glu	70 ± 9.9	72.2 ± 8.8	43.1 ± 11.7	0.2261
Gly	217.7 ± 31	256.3 ± 31	157 ± 36.7	0.1512
His	20.2 ± 2.4	21.6 ± 2.4	12.1 ± 2	0.0520
Ile	41.2 ± 4 [ab]	50.5 ± 4 [a]	27.3 ± 4.8 [b]	0.0075
Leu	78.9 ± 5.2 [a]	94.3 ± 5.2 [a]	51.2 ± 5.6 [b]	0.0001
Lys	81.7 ± 6.9 [a]	98.3 ± 6.9 [a]	52.5 ± 7.5 [b]	0.0013
Met	18.5 ± 1.6 [a]	17.9 ± 1.6 [a]	10.2 ± 1.8 [b]	0.0073
Orn	118.7 ± 13.1 [a]	111.2 ± 13.1 [ab]	65.9 ± 15.5 [b]	0.0468
Phe	47.9 ± 3.32 [ab]	59.4 ± 3.07 [a]	39.2 ± 3.32 [b]	0.0015
Ser	367.7 ± 39.7 [a]	413.1 ± 39.7 [a]	201.2 ± 42.9 [b]	0.0057
Tau	94.1 ± 16.3	82.9 ± 16.3	61.7 ± 17.6	0.4169
Thr	178 ± 21.1 [a]	147.2 ± 21.1 [a]	57 ± 25 [b]	0.0063
Trp	46.9 ± 2.7 [a]	46.7 ± 2.7 [a]	30.7 ± 3.28 [b]	0.0026
Tyr	46.4 ± 2.9 [a]	45.4 ± 2.9 [a]	30.1 ± 3.1 [b]	0.0027
Val	115.9 ± 7.4 [a]	127.8 ± 7.4 [a]	81.1 ± 7.9 [b]	0.0015
Total AA	2457.6 ± 145.6 [a]	2635.1 ± 145.6 [a]	1449.1 ± 172.3 [b]	0.0002

[1]Data is shown as $\bar{X}$±SEM. Different superscripts indicate significant difference between means.

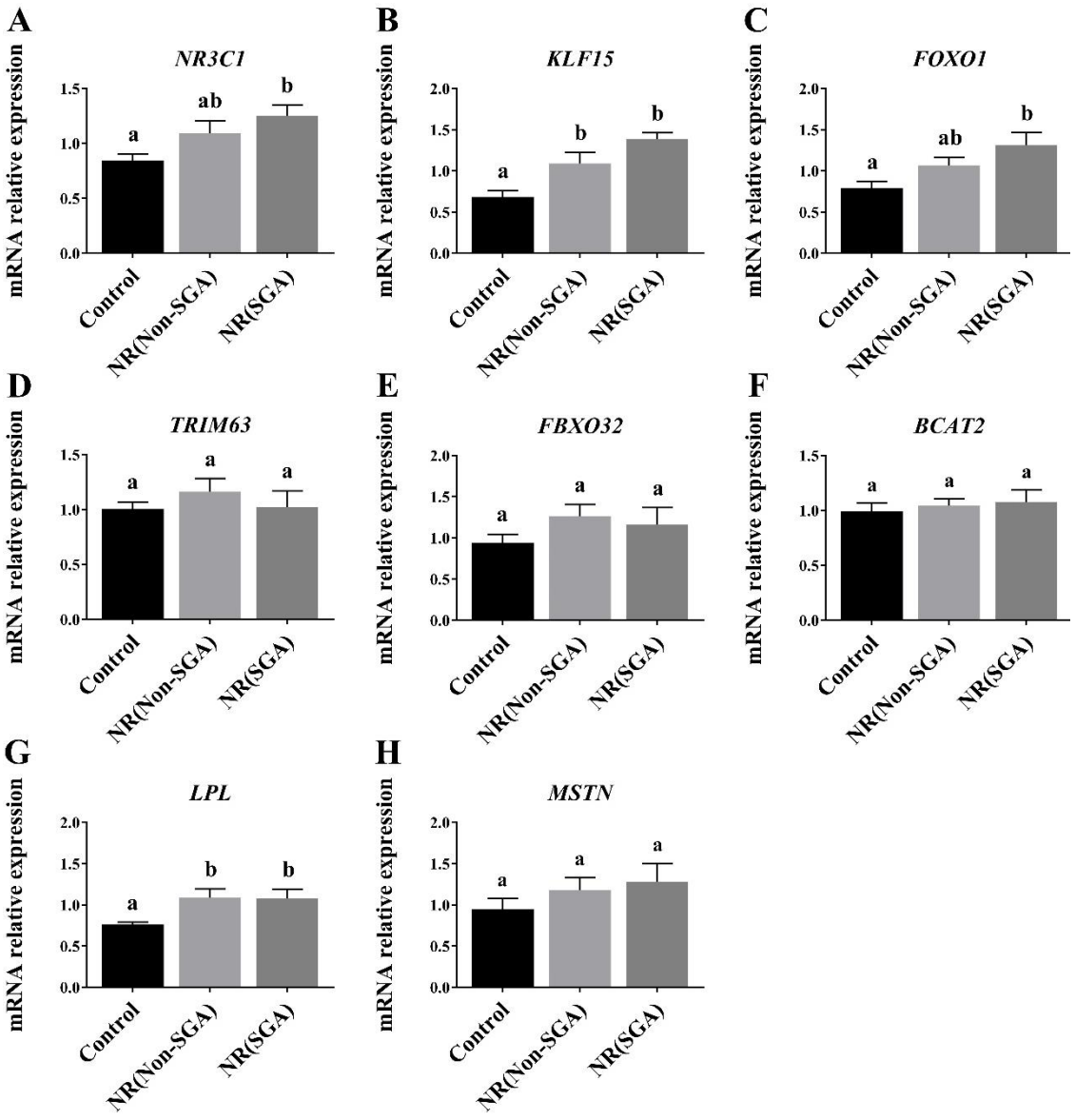

Figure 2.5 mRNA Expression in Gastrocnemius at GD 135. Relative mRNA expression for *NR3C1***(A),** *KLF15* **(B)**, *FOXO1* **(C)**, *TRIM63* **(D)**, *FBXO32* **(E)**, *BCAT2* **(F)**, *LPL* **(G)**, and *MSTN* **(H)** are shown. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ (P<0.05).

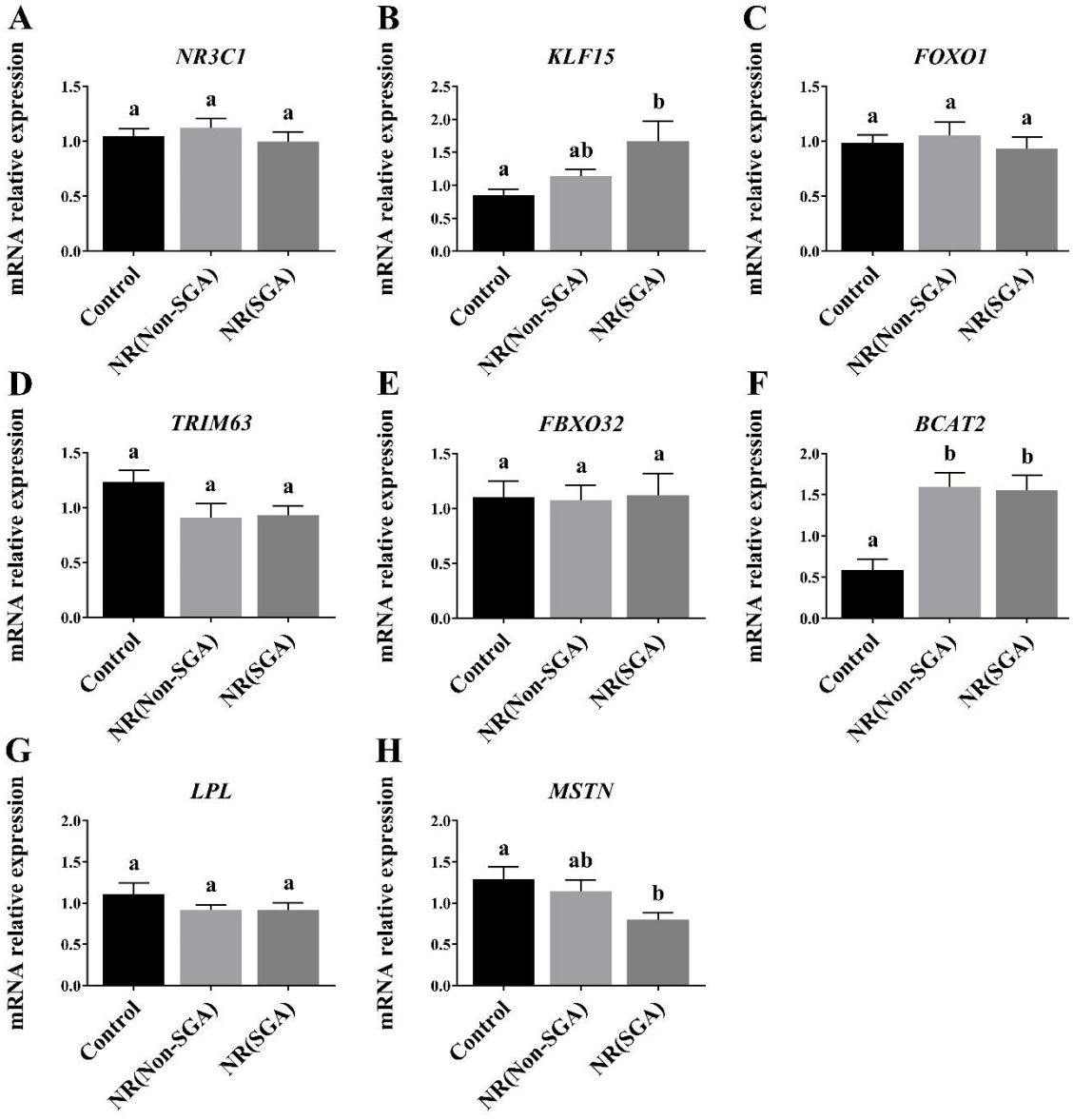

Figure 2.6 mRNA Expression in Soleus at GD 135. Relative mRNA expression for *NR3C1***(A)**, *KLF15* **(B)**, *FOXO1* **(C)**, *TRIM63* **(D)**, *FBXO32* **(E)**, *BCAT2* **(F)**, *LPL* **(G)**, and *MSTN* **(H)** are shown. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ (P<0.05).

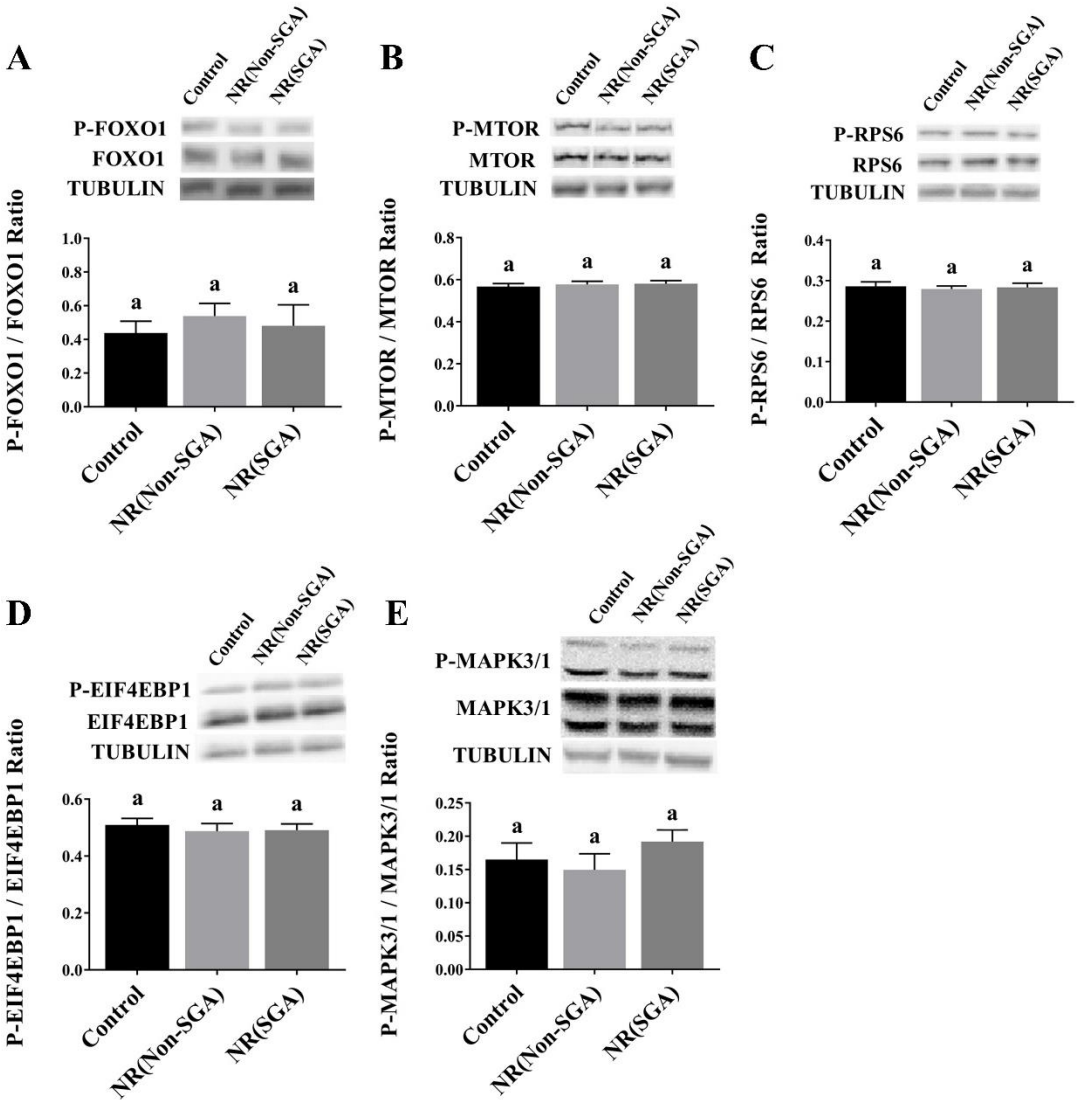

Figure 2.7 Protein Levels in Gastrocnemius at GD 135. Ratios between phosphorylated and total FOXO1 **(A)**, MTOR **(B)**, RPS6K **(C)**, EIF4EBP1 **(D)**, and MAPK3/1 **(E)** are shown. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.05$). Individual phosphorylated and total protein levels were not different for any of the target proteins (data not shown).

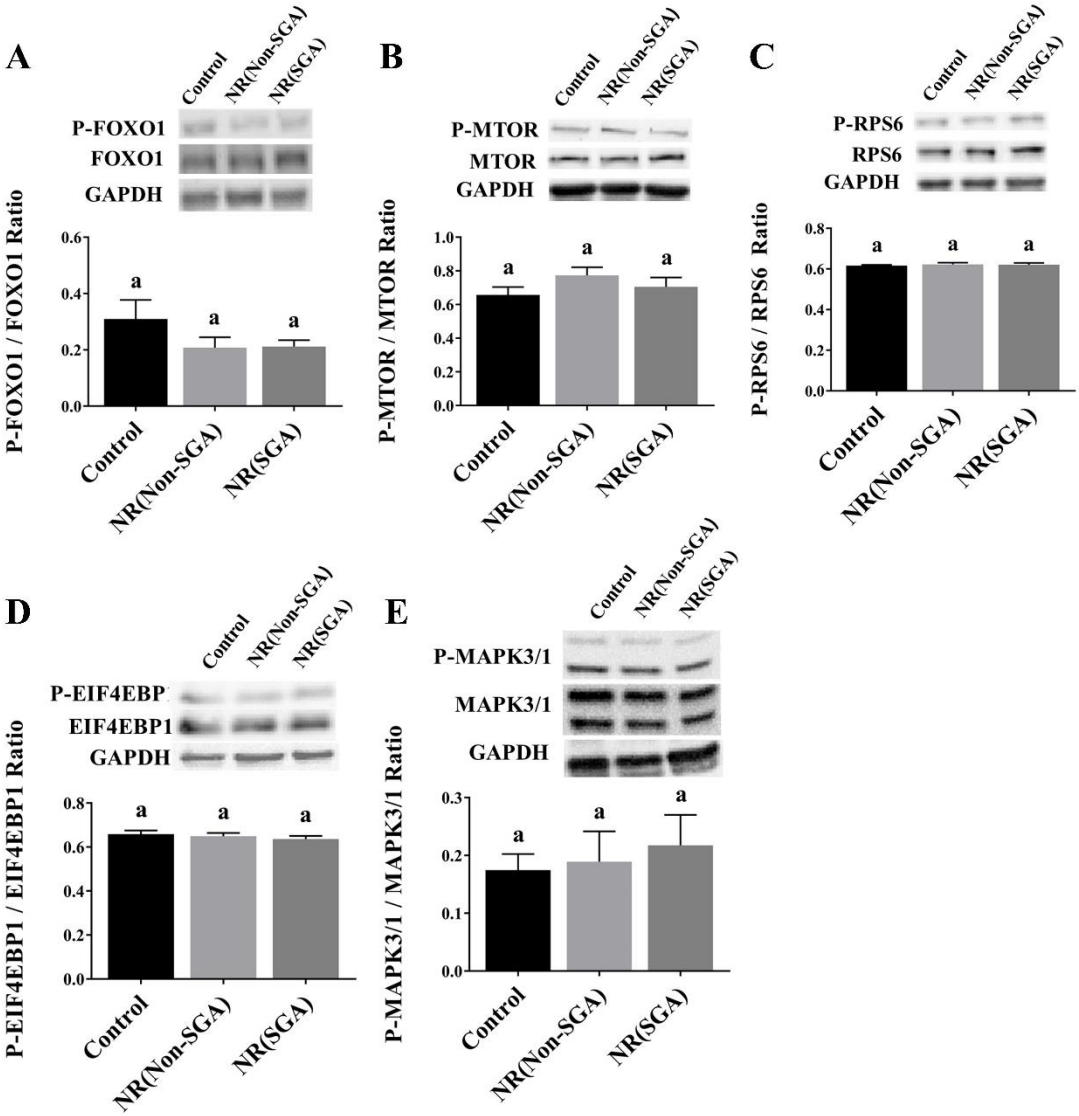

Figure 2.8 Protein Levels in Soleus at GD 135. Ratios between phosphorylated and total FOXO1 **(A)**, MTOR **(B)**, RPS6K **(C)**, EIF4EBP1 **(D)**, and MAPK3/1 **(E)** are shown. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.05$). Individual phosphorylated and total protein levels were not different for any of the target proteins (data not shown).

4. Discussion

The objective of this study was to evaluate the effect of differential responses to maternal NR on myofiber hypertrophy, glucocorticoid-mediated pathways for protein degradation, and its interactions with MTOR activation. These factors are determinants of fetal skeletal muscle growth, which if impaired, is associated with a greater predisposition to metabolic dysregulation in aged offspring. From an agricultural perspective, decreased lean mass in livestock species would negatively impact animal protein production, which would further affect human nutrition and health. Results highlight an absence of differences in pathways for protein synthesis and degradation in skeletal muscle, but enhanced availability of amino acids in the NR(Non-SGA) group, likely suggesting a unique materno-placental mechanism for amino acid transport in this group. This would support a higher rate of protein synthesis in the NR(Non-SGA) group and a suboptimal response in NR(SGA) fetuses likely due to lower precursor availability.

Several studies have previously described that fetal weight and skeletal muscle mass are decreased by maternal NR (Osgerby et al., 2002; Kwon et al., 2004; Fahey et al., 2005, Satterfield et al., 2009). Here we confirm our previous results of unequal alterations in fetal weight in response to our sheep model of maternal NR, as we continuously identify two phenotypes for fetal weight within the NR group, namely NR(SGA) and NR(Non-SGA). Likewise, we found that maternal NR does not necessarily impair skeletal muscle mass, as we have found reduced muscle weight in the NR(SGA) group compared to NR(Non-SGA) in both, gastrocnemius and soleus despite

exposure to the same prenatal treatment. However, for both muscles, myofiber sectional area was decreased in the NR(SGA) group compared to control but did not differ between NR(SGA) and NR(Non-SGA), with NR(Non-SGA) exhibiting an intermediate phenotype. These results indicate that alterations in myofiber hypertrophy do not completely explain the observed differences in muscle weight between NR(SGA) and NR(Non-SGA) groups.

Hypertrophy begins around GD 100 in the sheep (McCoard et al., 2001, Wei et al., 2014), and while it accounts for the majority of the increase in muscle mass, the maximum growth potential of this tissue is limited by myofiber number (Brown, 2014). Myofiber formation occurs in two main waves, primary myogenesis starting by GD 32 and secondary myogenesis starting by GD 65 in sheep (Maltin, 2008). Thus, our NR treatment from GD 35 to GD 135 would likely affect myofiber formation. A potential lower number of myofibers, in addition to decreased myofiber area, may explain the lower muscle weight observed for NR(SGA) at GD 135.

Muscle hypertrophy depends upon intracellular protein turnover, which will favor protein deposition if protein synthesis rates are higher than protein degradation rates. Results from a sheep model of fetal glucose restriction during the last 40% of pregnancy have shown an upregulation of mRNA expression of the ubiquitin ligases *FBXO32* and *TRIM63* in fetal skeletal muscle (Brown et al., 2014). These markers participate in the ubiquitin-proteasomal pathway, which is the main pathway responsible for myofibrillar protein degradation (Sandri, 2013). Interestingly, the study of Brown et al. (2014) found no upregulation in the autophagy-lysosome pathway; which also

accounts for a percentage of protein degradation in muscle and no downregulation in pathways for protein synthesis.

Glucocorticoids induce skeletal muscle catabolism and have been shown to upregulate FBXO32 and TRIM63 by binding to their receptor (NR3C1). Binding to the NR3C1 receptor upregulates expression of the starvation-sensitive factor KLF15 and FOXO1. KLF15 further induces transcription of FOXO1 and directly upregulates FBXO32 and TRIM63 (Shimizu et al., 2011). Cortisol is an endogenous glucocorticoid that plays an essential role in prepartum fetal maturation (Vaughan et al., 2018). It has also been demonstrated that fetal cortisol increases in response to intrauterine stressors such as hypoxia, without increases in maternal cortisol (Challis et al., 1989). These findings indicate responsiveness of the fetal adrenal gland to intrauterine stress conditions. However, the results of the present study indicate no differences in fetal cortisol concentration levels at GD135 in response to the nutritional stress imposed by our NR treatment.

In the gastrocnemius muscle, an upregulation in *NR3C1* mRNA was found in NR(SGA) compared to control. Having an upregulation in the receptor may increase glucocorticoid signaling regardless of the lack of differences in cortisol. We also observed an upregulation of *KLF15* mRNA expression in both NR(SGA) and NR(Non-SGA) groups compared to control. This suggests that upregulation in glucocorticoid signaling may have been achieved by upregulation of NR3C1.

Similarly, we found upregulation of *FOXO1* in NR(SGA) compared to control in gastrocnemius, further reinforcing an upregulation of NRC31 activity. However, there

were no differences in either total or phosphorylated protein levels of FOXO1, suggesting an absence of biological effect regardless of the upregulation at the gene expression level. This is consistent with our finding of no differences in mRNA expression of either *FBXO32* or *TRIM63* in gastrocnemius. For soleus muscle, there were no differences in *NR3C1*, while *KLF15* was upregulated in NR(SGA) compared to control, but neither *FOXO1, FBXO32*, nor *TRIM63* differed among groups. Hence, our results at the gene expression level do not provide supporting evidence for increased protein degradation mediated by muscle-specific ubiquitin proteins in either gastrocnemius or soleus muscles.

In addition to its role in protein degradation, KLF15 regulates energetic metabolism in skeletal muscle, inducing a switch towards fatty acid utilization in response to starvation (Takeuchi et al., 2016). This is mediated by enhanced lipid flux to skeletal muscle and upregulation of fatty acid oxidation (Haldar et al., 2012). Lipoprotein lipase (LPL) is an enzyme that releases fatty acids from circulating triglycerides for their absorption in different tissues (Kim et al., 2001). Here we measured expression of *LPL* as an initial indicator for fatty acid uptake capacity in fetal skeletal muscle in response to starvation. Results show an upregulation of LPL in both NR(SGA) and NR(Non-SGA) compared to control within gastrocnemius muscle. This may indicate a higher capacity for fatty acid uptake and metabolic programming in favor of fatty acid oxidation in this tissue. Interestingly, no differences in LPL expression were found in soleus, highlighting muscle-specific changes in gene expression.

Soleus and gastrocnemius muscles are expected to differ in metabolic characteristics because of their myofiber type composition. Soleus is formed primarily by type I myofibers while a mix between type I and type II myofibers is found in gastrocnemius. A mixed myofiber type composition provides higher metabolic plasticity between oxidative and glycolytic metabolism. Importantly, a majority of muscles in the body are closer to this mixed phenotype rather than the more homogenous phenotype of the soleus (Ogata and Mori, 1964; Maltin 2008). Thus, the upregulation of *LPL* in NR(SGA) and NR(Non-SGA) in gastrocnemius muscle may have an impact on whole-body metabolism. This is of particular relevance since it has been described that overexpression of LPL in skeletal muscle is associated with insulin resistance (Kim et al., 2001).

It is of special interest that LPL and KLF15 were upregulated in both, NR(SGA) and NR(Non-SGA) groups within gastrocnemius muscle. Low fetal weight has typically been associated with negative metabolic programming and was the feature characteristic used to determine experimental groups in the initial studies in the field (Barker et al., 1989). Our results add to a growing body of literature that having a normal fetal weight may not necessarily mean an absence of programming effect at the molecular level, which may lead to physiological alterations in postnatal life. Metabolic characterization at the skeletal muscle and whole-body level in NR(SGA) and NR(Non-SGA) was beyond the scope of the present study, but it warrants further research. Future findings may be of seminal relevance since the standard to classify fetuses as SGA or IUGR

depends primarily on fetal weight, but our current results suggest that this may not be completely accurate.

Another downstream effect of KLF15 activation is upregulation of BCAT2 (Shimizu et al., 2011), which mediates the first step of BCAA (leucine, valine, isoleucine) degradation in muscle. This has an impact on glucose homeostasis as BCAA are the primary donors of amino groups for the synthesis of alanine and glutamine in skeletal muscle, which are subsequently used as precursors in the liver (Garber et al., 1976). We found no differences in *BCAT2* expression within gastrocnemius muscle. However, there was an increase in total content of BCAA, and a higher concentration of leucine in NR(Non-SGA) fetuses compared with NR(SGA). This indicates an increased precursor availability for glutamine and alanine synthesis and release from skeletal muscle. Accordingly, there was a tendency towards increased concentrations of alanine and glutamine in NR(Non-SGA), and an increase in total contents of those amino acids in NR(Non-SGA) fetuses. This may support gluconeogenesis in these fetuses to counteract nutritional hardship. Activation of fetal gluconeogenesis has already been demonstrated in response to a chronic hypoglycemia model associated with placental insufficiency (Rozance et al., 2007).

In soleus muscle, there was an upregulation of *BCAT2* in both NR(SGA) and NR(Non-SGA) fetuses. This result can be interpreted as an attempt to protect glucose homeostasis in response to maternal NR, independent of fetal growth capacity. However, we anticipate that this may have a limited effect on whole-body metabolism since the

majority of muscles in the body are metabolically more similar to gastrocnemius than soleus muscle (Ogata and Mori, 1964).

A cross-talk between upregulation of BCAT2 and MTOR has been previously described (Shimizu et al., 2011). BCAA, particularly leucine, and arginine activate MTOR activity, which induces protein translation (Suryawan et al., 2008). Since BCAT2 degrades BCAA, the increase in its expression in soleus muscle may lead to inhibition of MTOR signaling. We also found decreased concentrations of leucine and arginine in fetal plasma of NR(SGA) fetuses compared to NR(Non-SGA), while total content of those amino acids was lower in the NR(SGA) group compared to both NR(Non-SGA) and controls. This suggests that downregulation of MTOR pathway is likely to occur in NR(SGA) fetuses. However, we found no differences in either total or phosphorylated MTOR in soleus muscle. Accordingly, there were no differences in either total or phosphorylated EIF4EBP1or RPS6, which are downstream targets of MTOR and induce protein translation when active. No differences were found in *BCAT2* mRNA expression, and total or phosphorylated MTOR, EIF4EBP1, and RPS6 in gastrocnemius. Additionally, for both soleus and gastrocnemius, there were no differences in total or active MAPK3/1, which is also involved in the stimulation of protein synthesis in skeletal muscle (Yuan et al., 2017).

Another negative regulator of muscle mass through MTOR inhibition is MSTN, which was downregulated in NR(SGA) compared to control group in soleus. We interpret this result as a potential muscle-specific attempt to compensate for low muscle mass in the NR(SGA) group. However, MTOR regulation is multifactorial, so

downregulation of a single negative regulator is less likely to induce a net effect, as demonstrated by the lack of differences in MTOR signaling found in this study. There were no differences in *MSTN* mRNA expression in gastrocnemius.

A previous study found a reduction in longissimus dorsi muscle weight and associated decrease in MTOR and RPS6KB1 protein phosphorylation in a sheep model of 50% NR between GD 28 and 78 (Zhu et al., 2004). Consistent with findings of the present study, however, we previously found no difference in MTOR signaling in the longissimus dorsi muscle at GD 125 using the same NR model as the present study (Keith et al., unpublished results). Differences in observed changes in MTOR may be explained by different timing of the insult or evaluation at an earlier GD. Nevertheless, the results of Zhu et al. (2004) showed no increase in ubiquitinated protein, which supports our results against increased protein degradation by upregulation of the ubiquitin-proteasomal pathway.

Intracellular protein turnover depends on the cycle of protein synthesis and degradation. Protein turnover is affected by several factors and will favor protein deposition when protein synthesis is enhanced over protein degradation. Examples of factors that will affect protein turnover are the availability of amino acids and energy substrates within a cell, and the presence of anabolic signals (Wu, 2013a). Our results do not support differences in pathways for protein degradation or synthesis between our experimental groups, regardless of decreased cross-sectional area in NR(SGA) fetuses. Protein synthesis is an energetically expensive process (Rattray and Joyce, 1976) that depends upon the availability of amino acids as precursors. It has been demonstrated that

50% maternal NR in the sheep reduced amino acid concentrations in maternal plasma and fetal plasma, allantoic and amniotic fluids (Kwon et al., 2004; Satterfield et al., 2009; Satterfield et al., 2013). Here we found a decrease in total amino acid concentration in NR(SGA) compared to NR(Non-SGA) and a decrease in amino acids total content in NR(SGA) compared to both NR(Non-SGA) and controls. This indicates a scarcity of building blocks for protein synthesis in NR(SGA) fetuses, which may limit this process regardless of MTOR signaling activation. This is supported by the study of Manjarin et al. (2018) which found that lower rates of protein synthesis in skeletal muscle of porcine neonates under energy and protein restriction, without downregulation of MTOR signaling. Considering that intracellular protein turnover is continuously occurring within the cell, limited protein synthesis will result in intracellular protein loss, and overall would impair myofiber hypertrophy.

Lower protein synthesis rates would affect both skeletal muscle and fetal weight as amino acids play an essential role in conceptus and fetal growth and survival (Lin et al., 2014). Interestingly, administration of sildenafil citrate to 50% NR ewes was effective to increase amino acids in fetal serum, allantoic and amniotic fluids, and prevented the appearance of SGA fetuses (Satterfield et al., 2009). This is a promising finding to prevent SGA offspring in NR pregnancies.

The increase in total content of amino acids in the NR(Non-SGA) group is of high relevance as it may indicate a unique materno-placental adaptation for enhanced amino acid transport in NR(Non-SGA) fetuses to counteract nutritional hardship. Moreover, plasma levels of arginine are increased in these fetuses. Arginine is a

precursor for polyamines and nitric oxide, and these molecules are essential in fetal growth because of their angiogenic activity which supports nutrient delivery to the fetus through placental tissues (Wu et al., 2009). Thus, increased arginine availability would further support an adaptation towards enhanced fetal growth in NR(Non-SGA) fetuses.

Arginine has also been positively associated with muscle growth by enhancing protein deposition (Tan et al., 2009) and it stimulates myoblast proliferation (Kalbe et al., 2013) and fusion (Long et al., 2006). Hence, it is possible that lower arginine in NR(SGA) group led to decreased myoblast proliferation, limiting muscle growth because of reduced myofiber formation. Also, decreased myoblast fusion capacity may lead to lower myonuclei content per myofiber, which would explain the decreased cross-sectional area seen in NR(SGA) group because each myonucleus can support a fixed amount of cytoplasm within a myofiber. Further studies are warranted to explore this possibility and to study the effect of our model in myofiber formation in NR(Non-SGA) and SGA fetuses.

CHAPTER III

EFFECT OF MATERNAL NUTRIENT RESTRICTION ON EXPRESSION OF GLUCOSE TRANSPORTERS (SLC2A1 AND SLC2A4) AND INSULIN SIGNALING MOLECULES IN SKELETAL MUSCLE OF SGA AND NON-SGA SHEEP FETUSES

1. Introduction

Maternal nutrient restriction (NR) is a cause of small for gestational age (SGA) offspring which exhibit accelerated growth in early postnatal life (De Blasio et al., 2007), and are at a higher risk of metabolic disease in adulthood (Barker and Clark, 1997; Ozanne and Hales, 1999). Associated metabolic diseases include insulin resistance (Ozanne and Hales, 1999; Gardner et al., 2005; Todd et al., 2009), type II diabetes (Hales and Barker, 1992; Rich-Edwards et al., 1999), cardiovascular disease (Barker et al., 1989), and obesity (Fernandez-Twinn and Ozanne, 2006). The thrifty phenotype hypothesis suggests that the onset of metabolic syndrome in response to maternal NR is programmed via fetal adaptations to nutrient scarcity. Affected fetuses would have modified pathways for nutrient uptake and storage, such as insulin signaling, and glucose transport and utilization (Hales and Barker, 1992) which may be beneficial during prenatal and early postnatal life. Nevertheless, if nutrient availability increases during postnatal life, there is a mismatch between the anticipated phenotype and the actual environment (Gluckman et al., 2010). This would lead to excessive nutrient

uptake, increased adiposity and intracellular fat infiltration, and transition into insulin resistance in aged offspring.

Fetal skeletal muscle is among the most susceptible tissues to maternal undernutrition because 'vital organs' are energetically prioritized in conditions of nutrient scarcity (Desai et al., 1996). Thus, skeletal muscle mass, metabolism, and myofiber type composition are likely to be altered by maternal NR. This has a detrimental effect on glucose metabolism since roughly 80% of insulin-mediated glucose uptake occurs in this tissue (Ferrannini et al., 1985). Impairment of skeletal muscle insulin sensitivity and glucose metabolism can lead to whole-body insulin resistance, which is one of the factors leading to type 2 diabetes and onset of metabolic syndrome (DeFronzo and Tripathy, 2009).

The main glucose transporter in fetal tissues, including fetal skeletal muscle, is solute carrier family 2 member 1 (SLC2A1) (aka GLUT1) (Santalucia et al., 1992) which is insulin-independent. Nevertheless, by the end of gestation solute carrier family 2 member 4 (SLC2A4) (aka GLUT4) starts to be upregulated in skeletal muscle and is the predominant glucose transporter in this tissue during postnatal life (Stuart et al., 2000). SLC2A4 is an insulin-dependent transporter which becomes active after translocation to the plasma membrane in response to activation of the PIK3CA/AKT pathway by insulin (Kohn et al., 1996). IGF1 can also trigger the activation of PIK3CA/AKT pathway (aka PI3K/AKT) by binding to its receptor or to INSRB (Mora et al., 1995; Alessi et al., 1996; Belfiore et al., 2009).

Previous research has shown an upregulation in molecules involved in insulin signaling and glucose transport in skeletal muscle of fetuses born to NR dams at the fetal or early postnatal stage (Ozanne et al., 1996; Costello et al, 2008; Lie et al, 2014), while insulin resistance has been reported in aged offspring (Gardner et al., 2005). An increase in type II myofibers has also been found in response to maternal NR (Zhu et al., 2006). An increase in type II myofibers would impair glucose metabolism because these myofibers have lower insulin sensitivity and oxidation capacity than type I myofibers (He et al., 2001). These results are consistent with the establishment of a thrifty phenotype at the skeletal muscle level and its progression to metabolic syndrome.

The vast majority of published studies have typically considered fetuses from NR dams as a single experimental group irrespective of their individual rates of fetal growth. In our studies, the NR sheep model yields a spectral phenotype of fetal growth rates, likely due to inherent abilities within the ewe to adapt to the nutritional hardship in an effort to support fetal growth. Due to these observations, we sought to characterize mechanisms by which the placenta is capable of adapting to nutritional hardship and the resultant responses by the fetus (Keith et al., unpublished results). The aim of the present study was to evaluate the effect of differential responses to maternal NR on the expression of glucose transporters (SLC2A1 and SLC2A4), insulin signaling molecules (INSRB, IGF1R, and AKT), and myofiber type composition.

2. Materials and Methods

All experimental procedures in this study were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

2.1 Animal Handling and Tissue Collection

Mature Hampshire ewes of similar frame size, parity, and body condition were fed to meet 100% of their nutritional requirements according to recommendations of the National Research Council (NRC) and used as embryo transfer recipients. A protocol for estrus synchronization and superovulation was administered to recipient and donor ewes. Hampshire donor ewes were artificially inseminated, and on gestational day (GD) 6, a single embryo was transferred from a donor ewe into the recipient uterus. Ultrasound was used as pregnancy diagnostic on GD 28. On GD 28, ewes were housed in individual pens on concrete and maintained in these conditions until GD 135. Ewes were fed once per day. Rations were adjusted once per week based on weekly measurements of body weight. On GD 35, ewes were randomly divided into a control-fed group which received 100% NRC requirements, and a nutrient-restricted (NR) group, which received 50% of total NRC requirements. Composition of the diet has been published previously (Lassala et al., 2010). Necropsies were conducted on GD 135. At this time, blood samples from fetal umbilical vein and fetal heart were collected to obtain fetal plasma. Soleus and gastrocnemius muscles were dissected and weighed, and samples preserved for further investigation.

Fetuses from ewes fed 100% NRC formed the **Control** group (n=12). Fetuses from NR ewes were divided into quartiles based on fetal weight distribution. The highest

(**NR(Non-SGA)** group; n=11) and lowest (**NR(SGA)** group; n=11) quartiles were selected for further investigation (Keith et al., unpublished results).

2.2 Glucose, Insulin, and IGF1 Concentration in Fetal Plasma

Glucose, insulin, and IGF1 concentrations in plasma were measured using, a glucose colorimetric kit (catalog number STA-680, Cell Biolabs, Inc., San Diego, CA), insulin ELISA (catalog number 80-INSOV-E01, ALPCO Diagnostics, Salem, NH), and IGF1 ELISA (catalog number SI0016, Neo Biolabs, Cambridge, MA) respectively. All kits were used according to manufacturer recommendations (Satterfield et al., 2013). To calculate total hormone and nutrient content in the fetal circulation, concentrations were multiplied by blood volume, which was estimated at 110ml/kg fetal weight (Brace, 1983; Brace, 1986).

2.3 Protein Extraction and Western Blots

Total protein from soleus and gastrocnemius muscles were extracted by tissue homogenization in lysis buffer using snap-frozen samples. Protein concentration was measured using Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as standard. Total INSRB (1:250, catalog number SC-81465, Santa Cruz Biotechnology, Dallas, TX), IGF1R (1:2000, catalog number ab182408, Abcam, Cambridge, MA), AKT and phosphorylated AKT (1:1000, catalog numbers 9271 and 9272, Cell Signaling Technology, Danvers, MA), SLC2A4 (1 µg/mL, catalog number ab33780, Abcam, Cambridge, MA), and SLC2A1 (1:50000, catalog number Ab115730, Abcam, Cambridge, MA) were quantified by western blots as previously described (Spencer et al., 1999) using 50 ug of protein. An IgG-horseradish peroxidase-conjugated

antibody (1:20000 of 1mg/ml stock) (KPL, Bethesda, MD) followed by incubation on SuperSignal™ West Dura (Thermo Scientific, Waltham, MA) were used to detect immunoreactive proteins by chemiluminescence. Proteins were quantified using Quantity One® 4.6.1 Software (Biorad Laboratories, Inc., Hercules, CA) with tubulin (1:10000, catalog number Ab7291, Abcam, Cambridge, MA) and GAPDH (1:10000, catalog number 2118, Cell Signaling Technology, Danvers, MA) as loading controls for soleus and gastrocnemius respectively.

2.4 Immunofluorescence

SLC2A4 translocation and myofiber type composition were evaluated by immunofluorescence. A modification of the protocol described by Wang et al. (2014) was used. Briefly, one section of 8 µm was obtained per sample using a cryostat at -16^{o}C, fixed in cold methanol for 10 minutes, and blocked using 10% normal goat serum for 1 hour at room temperature. After that, a cocktail of three antibodies against laminin (1:300, catalog number Ab44941, Abcam, Cambridge, MA), SLC2A4 (1:500, catalog number Ab33780, Abcam, Cambridge, MA), and Type I myosin heavy chain (1:75 for gastrocnemius, 1:50 for soleus, catalog number BA-D5, Developmental Studies Hybridoma, University of Iowa) was added for overnight incubation at 4°C. Mouse, rat, or Rabbit IgG were substituted for the primary antibody as a negative control. A cocktail of secondary antibodies (1:500, Alexa Fluor 488®, 594®, and 350®, Thermo Fisher Scientific, Waltham, MA) was then added for one hour of incubation at room temperature. Slides were overlaid using Prolong Antifade (Life Technologies, Carlsbad, CA) without DAPI.

Eight non-overlapping areas were imaged and evaluated per each section, using a Nikon Eclipse Ni-E fluorescence microscope and NIS-Elements AR 4.30.02 64-bit Software (Nikon Instruments Inc., Melville, NY). Colocalization between SLC2A4 and laminin was used as an indicator of SLC2A4 translocation. For myofiber type composition, any stained myofiber (blue) was identified as type I while non-stained myofiber was counted as type II. Results are expressed as Type I/Type II myofiber ratio.

2.5 Statistical Analysis

Data were analyzed using JMP® Pro 14 software (SAS Institute Inc., Cary, NC). One-way ANOVA ($\alpha = 0.05$) analysis was used to compare the means of control, NR(SGA), and NR(Non-SGA) groups. Differences between specific means were evaluated using Tukey's test.

3. Results

3.1 Glucose, Insulin, and IGF1 Concentration and Total Content in Fetal Plasma

We have previously reported that fetuses from NR ewes having SGA offspring were smaller ($P<0.05$) than fetuses from NR ewes having Non-SGA fetuses and control-fed females, which had similar weights to one another. Here we found no differences in either glucose concentration (mg/dL) ($P=0.3500$) or total glucose content (mg) ($P=0.2376$) in fetal plasma between groups (Figure 3.1A and 3.1D). Insulin concentration (ng/ml) in fetal plasma was lower ($P=0.0055$) in the NR(SGA) group than in the control group, while Non-SGA fetuses were not different from either NR(SGA) or control ($P>0.05$) (Figure 3.1B). Total circulating insulin was lower in NR(SGA) fetuses

compared to both control (P=0.0003) and NR(Non-SGA) (P=0.0422) groups (Figure 3.1E). IGF1 concentration in fetal plasma was not different between groups (P=0.2899) (Figure 3.1C). However, estimated total IGF1 levels were decreased in NR(SGA) group in comparison to control (P=0.0132), while NR(Non-SGA) was not different from either NR(SGA) or control (P>0.05) (Figure 3.1F).

3.2 Protein Expression

We have previously reported a reduction (P<0.05) in fetal gastrocnemius and soleus weight in NR(SGA) group compared to control and NR(Non-SGA) fetuses at GD 135. Here we found that within the gastrocnemius muscle, INSRB protein levels were greater in fetuses from the NR(SGA) in comparison to fetuses in the NR(Non-SGA) group (P=0.0285). INSRB content in fetuses from control fed ewes was not different between groups (Figure 3.2A). IGF1R protein levels were higher fetuses from the NR(SGA) group compared to fetuses from control-fed ewes (P=0.0059), while its content in fetuses from the NR(Non-SGA) group did not differ from either NR(SGA) or control (Figure 3.2B). Neither total nor phosphorylated AKT levels differed between groups (P=0.6500) (Figure 3.2C). SLC2A4 and SLC2A1 protein were higher (P=0.0110 and P=0.0383, respectively) in fetuses from the NR(SGA) group than fetuses from the NR(Non-SGA) group, while fetuses from the control group did not differ between either NR(SGA) or NR(Non-SGA) groups (Figure 3.3A and 3.3B).

In soleus muscle, there were no differences in INSRB (P=0.3690), IGF1R (P=0.5857), total AKT, phosphorylated AKT and their ratio (P=0.9042) (Figure 3.2D, 3.2E, and 3.2F), SLC2A4 (P=0.7125) and SLC2A1 (P=0.7090) (Figure 3.3C and 3.3D).

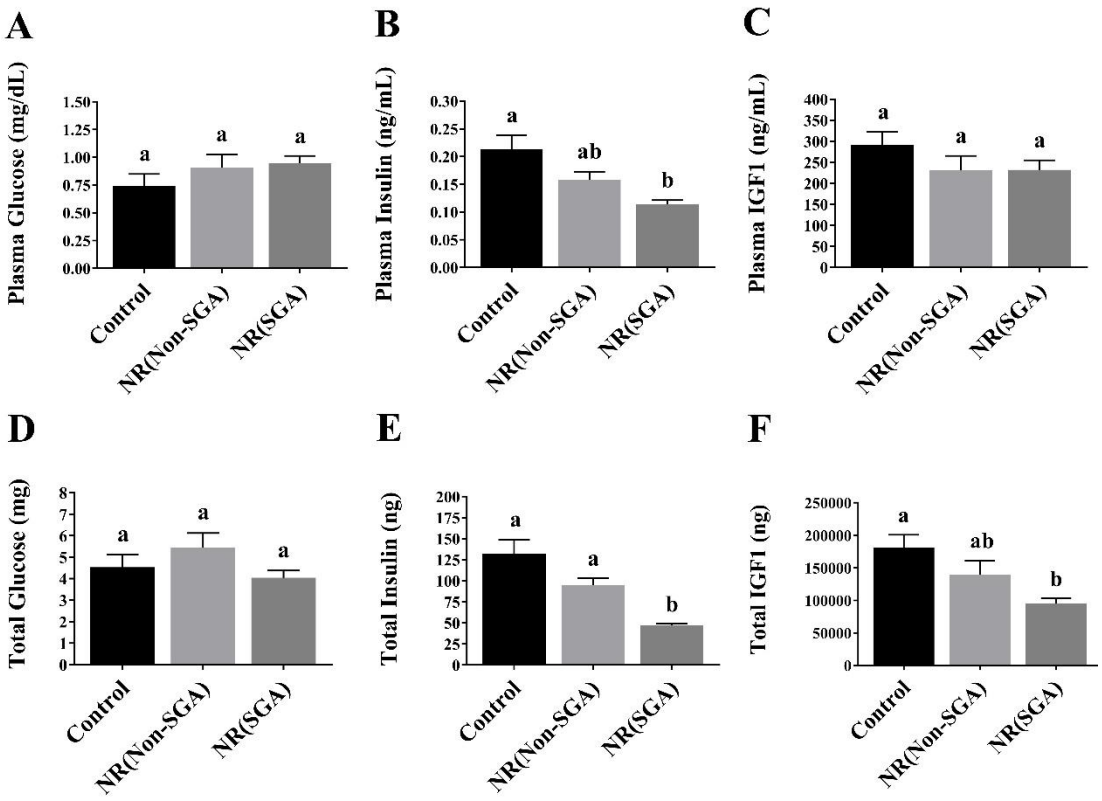

Figure 3.1 Plasma Concentration and Total Glucose, Insulin, and IGF1 at GD 135. **(A)**, **(B)**, and **(C)** show variations per group in fetal plasma concentrations of glucose, insulin, and IGF1, respectively. **(D)**, **(E)**, and **(F)** show estimated total content of glucose, insulin, and IGF1, respectively. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.05$).

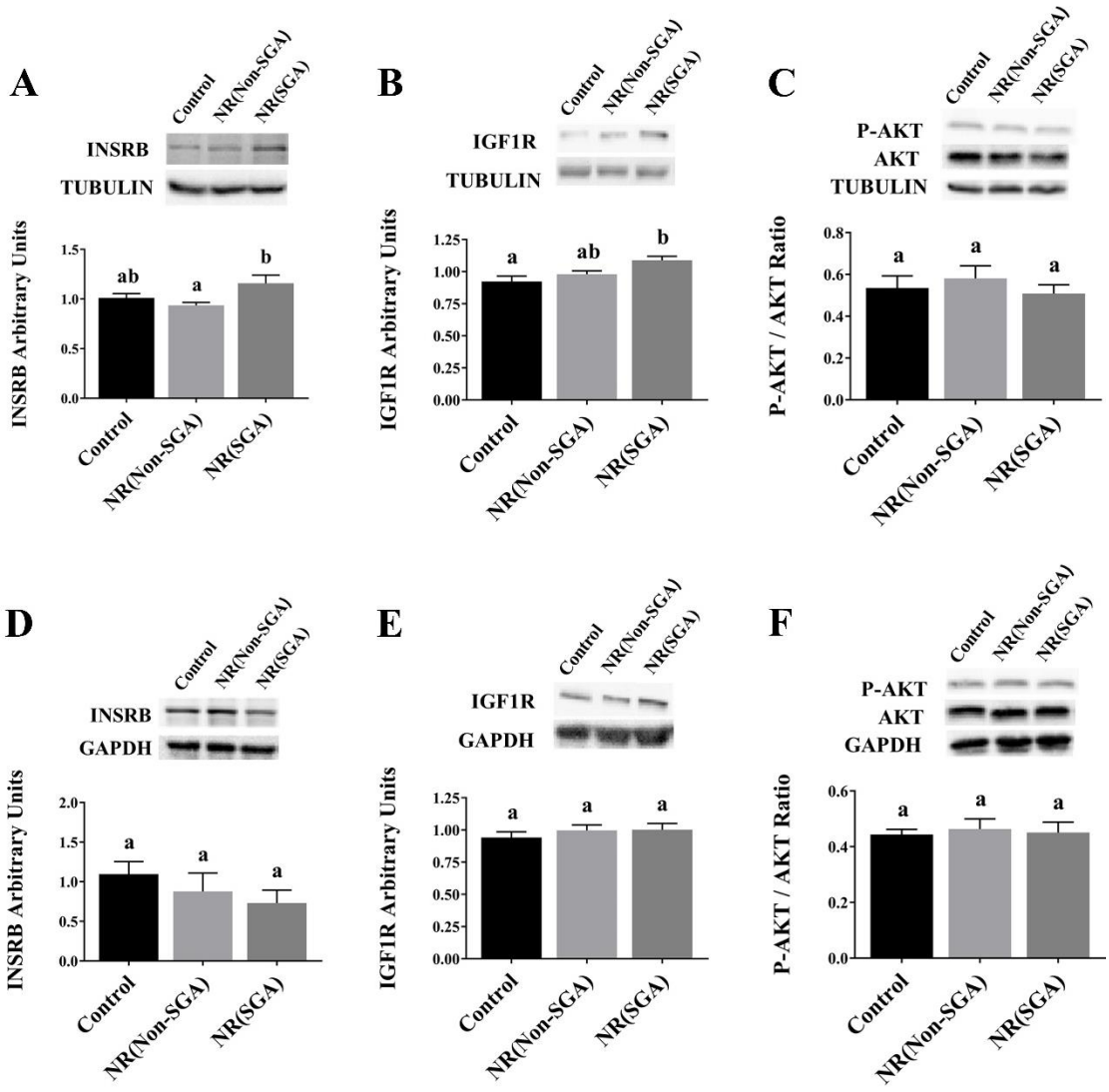

Figure 3.2 Protein Expression of PIK3CA/AKT Pathway Signaling Molecules in Fetal Muscles at GD 135. Insulin receptor β (INSRB), IGF1 receptor (IGF1R), and the ratio between total and phosphorylated AKT protein expression in gastrocnemius muscle are shown in **(A)**, **(B)**, and **(C)** respectively. INSRB, IGF1R, and the ratio between total and phosphorylated AKT levels in soleus muscle are shown in **(C)**, **(D)**, and **(E)** respectively. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.05$). Total and phosphorylated AKT did not differ in either gastrocnemius or soleus (not shown).

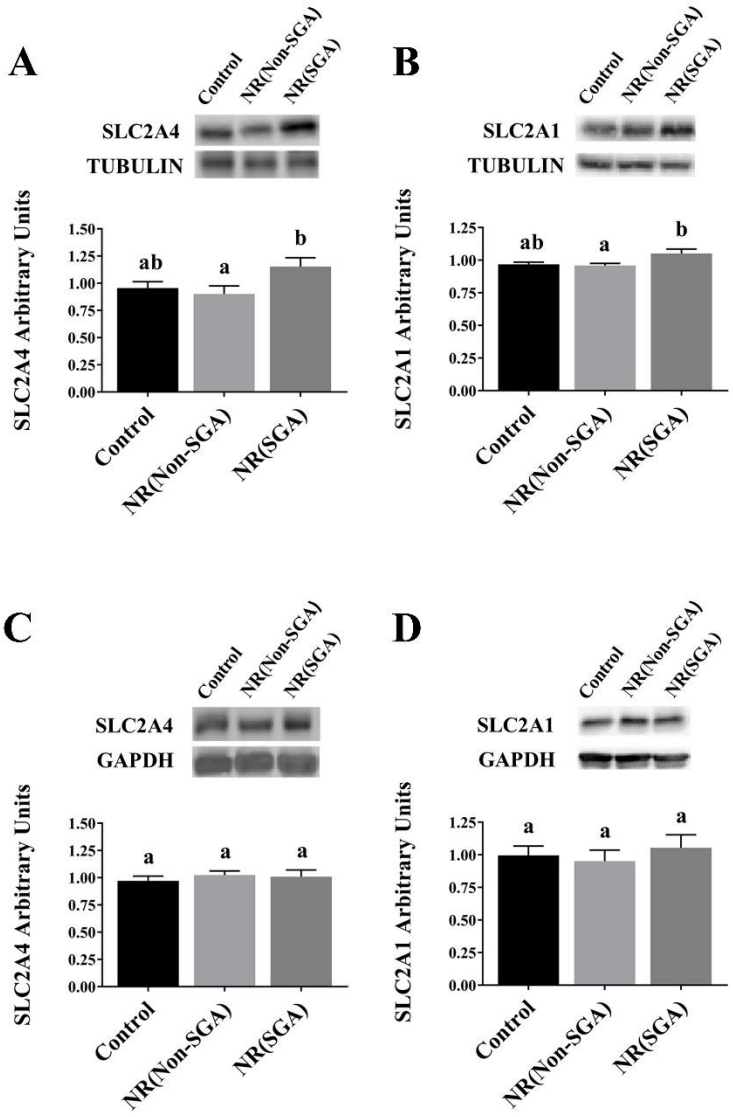

Figure 3.3 Protein Expression of Glucose Transporters in Fetal Muscles at GD 135. SLC2A4 and SLC2A1 protein levels in gastrocnemius muscle are shown in **(A)** and **(B)** respectively. SLC2A4 and SLC2A1 protein levels in soleus muscle are shown in **(C)** and **(D)** respectively. Data is shown as $\bar{X}$±SEM. Means without a common superscript between Control, NR(Non-SGA), and NR(SGA) differ ($P<0.05$).

3.3 SLC2A4 Translocation

For both, gastrocnemius and soleus (Figure 3.4A and 3.4B) muscles, SLC2A4 localization by immunofluorescence did not show observable differences among NR(SGA), NR(Non-SGA) and control groups. In addition, there was scarce overlapping between SLC2A4 and laminin staining, which indicates that there was no major translocation of SLC2A4 occurring at GD 135 in the fetus.

3.4 Myofiber Type Composition

The relative number of type I myofibers (Type I/Type II ratio) in gastrocnemius muscle was lower in fetuses from the NR(SGA) group compared to fetuses from both, NR (Non-SGA) (P=0.0046) and control (P=0.0196) groups. In the soleus muscle, fetuses from the NR(SGA) group had a lower Type I/Type II ratio than control (P=0.0145), while type I relative content was intermediate in NR(Non-SGA) fetuses and not different from either NR(SGA) or control fetuses (Table 3.1).

Table 3.1 Relative Myofiber Type Composition per Group in Gastrocnemius and Soleus Muscles at GD 135[1]

Tissue	Control (Type I/Type II)	NR(Non-SGA) (Type I/ Type II)	NR(SGA) (Type I/ Type II)
Gastrocnemius	0.25 ± 0.002^{a}	0.26 ± 0.002^{a}	0.16 ± 0.002^{b}
Soleus	0.22 ± 0.016^{a}	0.19 ± 0.016^{ab}	0.16 ± 0.016^{b}

[1]Means represent the ratio between type I and type II myofibers in Control, NR(Non-SGA), and NR(SGA). Data is shown as $\bar{X} \pm$SEM. Different superscripts indicate $P<0.05$.

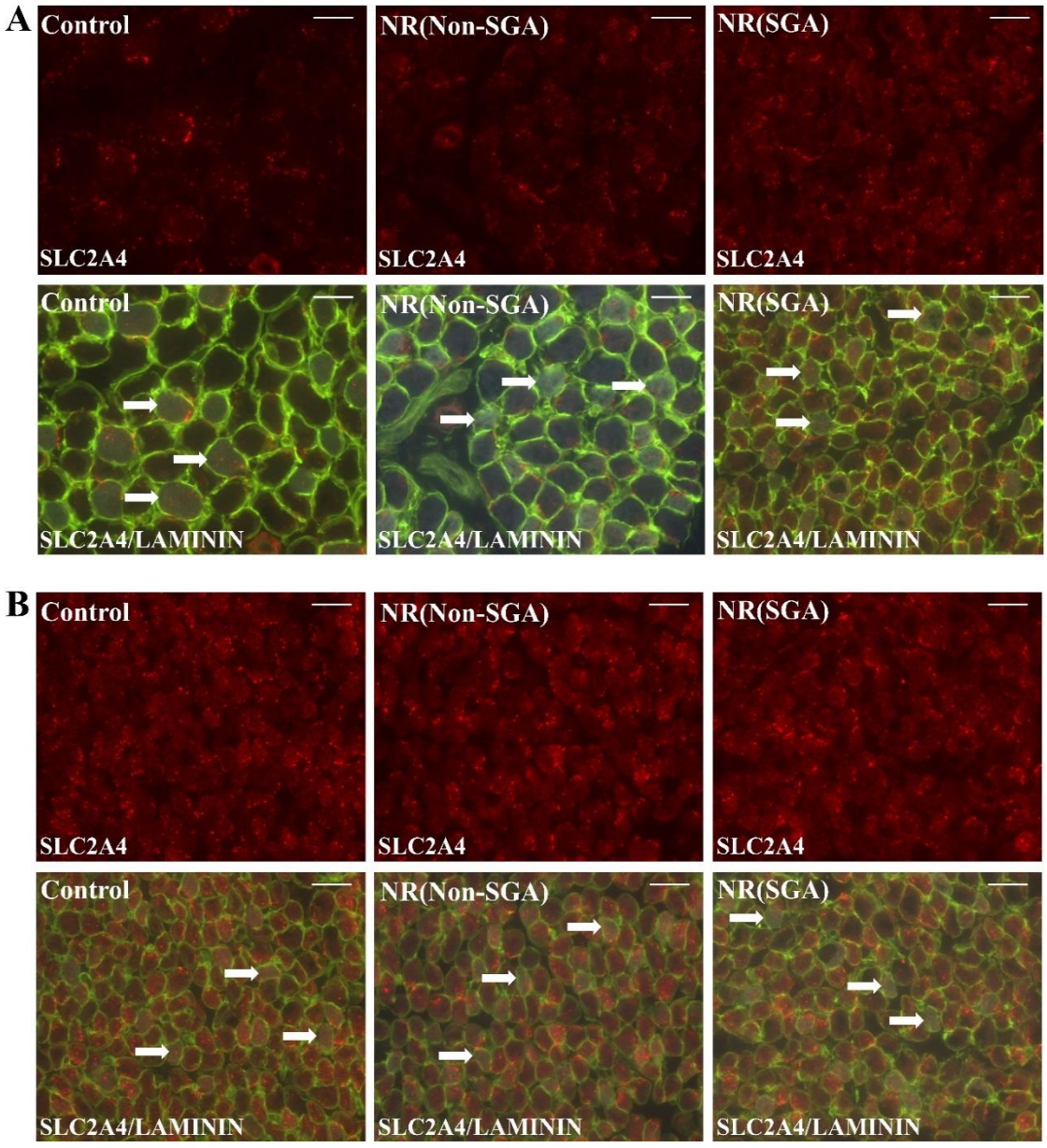

Figure 3.4 SLC2A4 Localization and Myofiber Type Identification in Fetal Muscles at GD 135. Immunoreactive proteins for SLC2A4 (Red), Laminin (Green), and Type I Myosin Heavy Chain (Blue) are shown for Gastrocnemius (A) and Soleus (B). White arrows indicate examples of Type I myofibers. Laminin was used as a plasma membrane marker to colocalize SLC2A4 and evaluate its translocation. For the IgG control (not shown), normal rabbit, rat, and mouse IgGs were substituted for the primary antibody, respectively. Pictures were taken at 40X magnification. Scale bar equals 25 µm.

4. Discussion

The objective of the present study was to evaluate the effect of differential responses to maternal NR on glucose transporters, insulin signaling molecules, and myofiber type composition in fetal skeletal muscle, which could set metabolic alterations that ultimately lead to increased risk for disease during postnatal life. Results highlight molecular differences between SGA and Non-SGA fetuses receiving the same prenatal NR treatment, likely suggesting a differential ability of the dam to support fetal growth during hardship which may convey resistance to metabolic disease in the adult offspring. This study also confirms previous findings regarding the relative resistance of the fetal soleus muscle to maternal NR (Ward and Stickland, 1991 and Costello et al., 2008) as the only affected characteristic in this muscle was myofiber type composition.

Insulin is an anabolic hormone involved in glucose and amino acid uptake in the body (Dimitriadis et al., 2011). During the fetal stage, insulin in the fetal circulation is positively correlated with fetal growth (Fowden et al., 1989). It has been previously found that maternal nutrient restriction from GD50 to term induces a reduction in fetal insulin (Vonnahme et al., 2010). Similarly, the present study showed a decrease in plasma insulin concentration in NR(SGA) fetuses compared to control, and a marked reduction in total insulin in NR(SGA) fetuses compared to both, NR(NonSGA) and control. Lower insulin levels in NR(SGA) group suggest a reduction in anabolic signals in these fetuses, which is manifest in the reduction in fetal growth seen in this group, and likely indicate increased risk for alterations in glucose metabolism and development of metabolic syndrome in adulthood.

Circulating glucose levels are the main trigger for insulin secretion from pancreas (Hellman et al., 1994). This study found no effects of maternal NR on fetal glucose plasma concentration or estimated total; hence, hypoglycemia was not the cause for the reduced insulin content in NR(SGA) group. Other nutrients, such as amino acids have also been found to induce insulin secretion (Floyd et al., 1966; Newsholme et al., 2006). Particularly leucine, a BCAA, and arginine can induce pancreatic insulin release (Schmidt et al., 1992; Kalogeropoulou et al., 2008). Using a model of 50% maternal NR, it has been previously described that concentrations of arginine, branched-chain amino acids (BCAA), and total amino acids are reduced in fetal plasma, amniotic and allantoic fluids at GD135 (Kwon et al., 2004).

Similarly, we have previously described lower concentration and total content of arginine and leucine in NR(SGA) fetuses. Thus, a decrease of circulating amino acids in response to maternal NR cannot be ruled out as a cause for impaired insulin release in this group. Altered capacity of the pancreatic beta-cell to release insulin has previously been described in intrauterine growth-restricted fetuses induced by placental insufficiency (Limesand et al., 2006). Likewise, 50% maternal NR has been associated with decreased pancreatic beta-cell mass in a rat model (Garofano et al., 1997), and a similar response could have been induced by our treatment. Interestingly, it has been demonstrated that administration of arginine to 50% NR ewes is effective to enhance fetal pancreas growth (Satterfield et al., 2013). In addition, sildenafil citrate treatment has been shown to increase the availability of amino acids and fetal growth in fetuses under 50% NR (Satterfield et al., 2009). These results represent potential interventions to

counteract the negative effect of maternal NR on fetal and pancreas growth, likely decreasing the risk of metabolic disease in adulthood.

Insulin exerts its anabolic effect in part by upregulating the PIK3CA/AKT pathway after binding to its receptor. Activation of AKT induces translocation of SLC2A4, which stimulates insulin-mediated glucose uptake. Upregulation of INSRB protein content (Gardner et al., 2005) and mRNA expression (Costello et al., 2008) have been previously described in sheep fetal skeletal muscle in response to maternal NR. Likewise, we found that within gastrocnemius, INSRB protein content was upregulated in the NR(SGA) group compared to NR(Non-SGA). This indicates that results from our NR(SGA) group are concordant with previous findings for NR fetuses. More importantly, this suggests that our NR(Non-SGA) group may represent a unique phenotype that is not equally affected the maternal NR in terms of fetal weight, and likely will not experience the same metabolic dysregulations than NR(SGA) in adulthood. However, is still unknown until which extent the NR(Non-SGA) group is protected from maternal NR programming, or how the potential adaptations that support fetal growth in early life may lead to other negative health outcomes during postnatal life.

We interpret our finding of increased INSRB in NR(SGA) group as an attempt to compensate for lower insulin availability. This possibility is supported by the lack of differences seen in total, or phosphorylated (active) AKT, or their ratio, in this study. AKT activation can also stimulate the MTOR pathway for protein synthesis (Boucher et al., 2014). We have previously indicated that our model does not induce differences in

MTOR pathway, and our current results for AKT further support that finding. AKT can also be phosphorylated in response to other ligands that activate the PIK3CA/AKT pathway, such as IGF1 (Manning and Toker, 2017). Thus, a potential amplification of insulin signaling by upregulation of INSRB is not the only possible cause for the absence of differences in active AKT.

Additionally, the upregulation of INSRB seen in this study could have a role in the short period of enhanced insulin sensitivity that has been suggested to happen in early postnatal life of SGA offspring, before progression towards insulin resistance in adulthood (Geremia and Cianfarani, 2004; Ibáñez et al., 2006; Morrison et al., 2010). The present study also observed a reduction of total IGF1 in NR(SGA) group compared to control, while NR(Non-SGA) had intermediate levels and did not differ from the other two groups. IGF1 has an anabolic role in fetal growth as its concentration shows a positive correlation to fetal size (Verhaeghe et al., 1993) and its deficiency can induce intrauterine growth retardation (Baker et al., 1993). Moreover, an intraamniotic administration of IGF1 has been shown to increase fetal growth in a model of prenatal growth restriction induced by placental embolization (Wali et al., 2012). Thus, as previously discussed for insulin, a decrease of total IGF1 in the NR(SGA) group is likely one of the alterations correlated to the intrauterine growth retardation in these fetuses. IGF1 exerts its action by binding to IGF1R preferentially, and to insulin receptor but with lower affinity (Belfiore et al., 2009). This growth factor can also activate the PIK3CA/AKT pathway in several tissues, including skeletal muscle (Schiaffino and Mammucari, 2011). Interestingly, in gastrocnemius muscle, IGF1R was upregulated in

the NR(SGA) group compared to controls. This follows a similar pattern than previously described for insulin and INSRB and further supports the establishment of a compensatory response in fetuses from the NR(SGA) group to account for the circulating levels of anabolic hormones.

SLC2A4 is among the most abundant glucose transporters expressed in skeletal muscle (Stuart et al., 2000), and its action is insulin dependent (James et al., 1988). Activation of the PIK3CA/AKT pathway induces SLC2A4 translocation to the sarcolemma where it exerts its glucose transport function. Previous studies have demonstrated an upregulation of *SLC2A4* mRNA in skeletal muscle of sheep fetuses in response to a maternal NR treatment applied during late gestation (Costello et al., 2008) or the preimplantation period (Lie et al., 2014). Similarly, results of the present study found an upregulation of SLC2A4 protein content in NR(SGA) compared to NR(Non-SGA) fetuses within gastrocnemius muscle, suggesting a compensatory response to the reduction in insulin. However, the lack of evident differences in SLC2A4 translocation among groups suggests that there is probably no net effect on glucose transport at GD135 in this specific tissue.

During postnatal life, an increase in nutrient availability may increase insulin secretion to induce SLC2A4 translocation. Exercise also stimulates translocation of this glucose transporter, and its effect would become relevant postnatally by incorporation of locomotion (Garg et al., 2009). Thus, we anticipate that under postnatal conditions the upregulation in SLC2A4 will lead to enhanced glucose uptake in skeletal muscle of NR(SGA) fetuses, which is one possible cause contributing to the accelerated growth

that is typically seen postnatally in SGA fetuses (De Blasio et al., 2007). Likewise, we found the same expression pattern for SLC2A1, which is an insulin-independent glucose transporter that is expressed at lower levels than SLC2A4 in adult skeletal muscle, but it is highly expressed in fetal tissues (Santalucia et al., 1992). This would further boost glucose uptake capacity within gastrocnemius muscle in NR(SGA) fetuses.

In soleus muscle, there was no upregulation in either INSRB, IGF1R, AKT, SLC2A4, or SLC2A1. These results support the relative resistance of this muscle to prenatal undernutrition, which has previously been described in the literature (Ward and Stickland, 1991; Costello et al., 2008). The majority of myofibers in the soleus are formed during primary myogenesis which occurs in early pregnancy, likely been relatively protected from the effects of maternal NR. However, most of the developing muscles, including the gastrocnemius, are composed of a higher proportion of secondary myofibers, which begin to form by GD 62 in the sheep and are more prone to be affected by maternal NR (Ward and Stickland, 1991). Also, in the adult organism, most of the muscles, including gastrocnemius, are formed by a mix of type I and type II myofibers, instead of almost exclusively having one of them, while soleus is primarily composed of type I myofibers once it acquires expression of adult myosin heavy chain (Ogata and Mori, 1964; Maltin, 2008). This suggests that our findings within gastrocnemius muscle are more likely to represent the response of most muscles in the body, which may lead to a whole-body metabolic programming effect.

Myofiber type composition influences muscle metabolism because it has an impact on insulin sensitivity, as well as in nutrient utilization and oxidative capacity (He

et al., 2001). Specifically, type I myofibers are oxidative and have higher insulin sensitivity than type II myofibers. A decreased proportion of type I myofibers has previously been found at GD 127 in fetal triceps brachii muscle in response to 50% NR in sheep (Costello et al., 2008). Likewise, our results indicate a lower proportion of type I myofibers in NR(SGA) group compared to both, control and NR(Non-SGA) in gastrocnemius muscle. Similarly, for soleus muscle, type I myofiber proportion was lower in NR(SGA) compared to control, while NR(Non-SGA) showed an intermediate proportion and did not differ from the other two groups.

Poly-neuronal innervation of myofibers has been described at the fetal stage, which may lead to co-expression of characteristics of different myofiber types in the same cell (Redfern, 1970). Poly-neuronal innervation disappears by the second week of postnatal life in rats (Brown et al., 1976), and likely during late pregnancy in precocial species such as the sheep. Hence, prenatal observations regarding myofiber type composition may not represent the adult stage, but if our results for myofiber type composition are maintained in postnatal life, it could impair insulin sensitivity at the skeletal muscle level, primarily in the NR(SGA) group, which could lead to whole-body metabolic alterations in glucose homeostasis. This is supported by previous research suggesting that an increased proportion of type II myofibers is associated with insulin resistance in skeletal muscle (Nyholm et al., 1997), and that altered insulin sensitivity in peripheral tissues is among the first noticeable alterations in the progression to insulin resistance and type 2 diabetes (Warram et al., 1990).

Interestingly, a treatment of 50% NR from GD 28 and GD 78 in sheep has been associated to increased content of type IIb myofibers in longissimus dorsi of 8-month-old offspring (Zhu et al., 2006). This suggests a long-lasting effect of prenatal NR on myofiber composition. However, contradictory results have been found in longissimus dorsi of 14-day-old lambs in which an increase in type I myofibers was found after 50% NR from GD 30 to 70 (Fahey et al., 2005). This divergence may be due to the differences in the treatment, or muscle used in the study. Also, postnatal conditions may influence results as myofiber type composition conserves a certain level of plasticity during postnatal life in response to some stimuli such as exercise. Thus, more research is still needed to confirm the long-lasting effect of myofiber type programming during fetal development (Brown, 2014).

Previous research suggests that prenatal programming for magnification of insulin-mediated glucose uptake sets up the conditions for insulin resistance in adult offspring (Costello et al., 2008). Intramuscular triglyceride (IMTG) accumulation is a cause for disruption in insulin signaling, and insulin resistance in skeletal muscle (Corcoran et al., 2007). We have previously found increased mRNA expression of lipoprotein lipase (LPL) in gastrocnemius muscle in both, NR(SGA) and NR(Non-SGA) groups compared to control. LPL is an enzyme that releases fatty acids from circulating triglycerides and its overexpression in skeletal muscle has been related to insulin resistance (Kim et al., 2001). Increased fatty acid uptake, together with lower oxidative capacity given the decreased type I myofiber proportion in NR(SGA) group, would lead

to IMTG accumulation, and a possible cause for the onset of insulin resistance during later life of NR(SGA) offspring. Further research is required to test this hypothesis.

CHAPTER IV

SUMMARY AND CONCLUSIONS

1. Summary

The initial studies on fetal programming identified epidemiological correlations between maternal undernutrition, low birth weight of the offspring, and increased risk for metabolic syndrome during adulthood (Barker 1989; Hales and Barker, 1992). These observations have been confirmed using animal models of maternal nutrient restriction to induce small for gestational age offspring (SGA). Several fetal organs are affected in response to maternal NR (Osgerby et al., 2002; Vonnahme et al., 2003; Lloyd et al., 2012; Satterfield et al., 2013). Among them, skeletal muscle is of particular relevance because of its implications in glucose and oxidative metabolism, and because in an agricultural context, it has an impact on animal protein production for human consumption.

Several authors have studied the effect of maternal NR on skeletal muscle (Zhu et al., 2004; Quigley et al., 2005; Fahey et al., 2005; Ford et al., 2007; Costello et al., 2008; Lie et al., 2014). The vast majority of published studies have considered fetuses from NR dams as a single experimental group. However, our observations consistently indicate that the NR sheep model yields a spectral phenotype of fetal growth rates, likely due to inherent abilities within the ewe to adapt to the nutritional hardship and support fetal growth. Due to these observations, we sought to characterize mechanisms by which the placenta is capable of adapting to nutritional hardship and the resultant

responses by the fetus. Hence, studies were conducted to characterize the response of fetal skeletal muscle to maternal NR using NR(SGA) and NR(Non-SGA) fetuses as experimental groups.

We have confirmed our previous findings of unequal alterations of fetal weight in response to maternal NR and observed the same pattern for individual muscle weights in both, gastrocnemius and soleus muscles at GD 135. In CHAPTER II, we aimed to study the differences in skeletal muscle growth in terms of hypertrophy, and found that a decrease in myofiber cross-sectional area (CSA) in the NR(SGA) group was not associated with markers for upregulation in pathways for proteasomal protein degradation (glucocorticoid-KLF15-ubiquitin ligases pathway) or downregulation in pathways for protein synthesis (MTOR pathway). This last finding was supported by the lack of differences found in total or active AKT, which is a positive regulator of MTOR signaling (CHAPTER III).

However, activation of MTOR signaling molecules is not necessarily associated with a sustained rate of protein synthesis under energy and protein restriction conditions (Manjarin et al., 2018). Interestingly, we found increased total content and concentration of total amino acids in NR(Non-SGA) group compared to NR(SGA) (CHAPTER II). Based on our observations, we suggest that an overall lack of amino acid availability in NR(SGA) fetuses would impair protein synthesis rates due to a reduction in available building blocks, without necessarily leading to a downregulation in markers of MTOR activation. This would explain, at least in part, the lower CSA found for NR(SGA) fetuses in both, gastrocnemius and soleus muscles, and would also have an overall

detrimental effect on fetal growth. The fact that NR(Non-SGA) fetuses had higher availability of amino acids compared to NR(SGA) is of seminal relevance as it may indicate a unique maternal-placental adaptation to enhance amino acid transport in an attempt to counteract nutritional hardship.

Muscle weight at GD 135 may have also been affected by a lower number of myofibers. Studying myofiber formation was beyond the scope of the present studies because it occurs at an earlier gestational age, but our NR treatment from GD 35 to GD 135 had the potential to impair myofiber formation. Of particular interest is the fact that concentration and total content of the amino acid arginine were reduced in NR(SGA) fetuses (CHAPTER II). Arginine is essential to promote placental, fetal, and skeletal muscle growth. It also has a positive effect on myoblast proliferation and fusion, which are relevant processes in myofiber formation. Thus, reduced arginine in the NR(SGA) fetuses may be correlated with a potential decrease in total myofiber dotation, which will further limit muscle growth in gastrocnemius and soleus muscles.

Stimulation of fetal and skeletal muscle growth depends upon anabolic signals. Some amino acids, such as arginine and leucine have a stimulatory effect on the release of insulin, which is an anabolic hormone (Schmidt et al., 1992; Kalogeropoulou et al., 2008). Here we found that both, concentration and total content of arginine and leucine were increased in NR(Non-SGA) compared to NR(SGA) fetuses (CHAPTER II). Accordingly, when we aimed to characterize the effect of our treatment on pathways for glucose uptake (CHAPTER III), we found lower total insulin in NR(SGA) fetuses without differences in glucose concentrations or total content. This suggests that amino

acid signaling for insulin release may be impaired in NR(SGA) fetuses, leading to downregulation of anabolic signals which would affect both, fetal skeletal muscle and whole-body growth. Assessment of pancreas developmental programming is beyond the scope of the present studies, but alterations in this organ are also a likely cause for lower insulin levels in NR(SGA) fetuses.

We also found lower total content of IGF1 in NR(SGA) fetuses compared to control, and interestingly there was an upregulation of both, INSRB and IGF1R in this group within gastrocnemius muscle (Chapter III). We interpret this result as an attempt to mount a compensatory response to counteract the lower content of anabolic hormones. This may be beneficial to sustain fetal life under nutrient scarcity conditions. SLC2A4 and SLC2A1 were also upregulated within NR(SGA) compared to NR(Non-SGA) fetuses within gastrocnemius muscle. We anticipate that during early postnatal life, the upregulation of insulin and IGF1 receptors, SLC2A4, and SLC2A1 would provide the molecular basis for initial accelerated postnatal growth and establishment of a thrifty phenotype.

Previous authors have indicated that upregulation of insulin signaling at the fetal stage would increase the risk of insulin resistance in postnatal life (Costello et al., 2008). Accumulation of intramuscular triglycerides is a cause for impairment in insulin signaling (Corcoran et al., 2007) and here we found an upregulation of *LPL* in both NR(SGA) and NR(Non-SGA) fetuses within gastrocnemius muscle. Similar results were found for *KLF15* (CHAPTER II). In addition to the role of KLF15 in glucocorticoid-mediated muscle atrophy, it plays a role in metabolic regulation in response to

starvation. One of the described effects is an increase in lipid utilization in skeletal muscle in an attempt to switch metabolism towards fatty acid oxidation and spare glucose. Thus, our results for *KLF15* and *LPL* may indicate an enhanced lipid uptake in fetal skeletal muscle in response to starvation. However, oxidative capacity may be specifically impaired in NR(SGA) fetuses because of a lower proportion of type I myofibers. If these conditions are maintained during postnatal life, increased uptake of fatty acids together with decreased oxidative capacity may lead to lipid accumulation, and be a potential cause for insulin resistance during adulthood in NR(SGA) offspring.

The fact that *LPL* and *KLF15* were upregulated in both, NR(SGA) and NR(Non-SGA) is of particular relevance as it suggests that NR(Non-SGA) is not completely protected from the programming effect induced by maternal NR. This is supported by results for *BCAT2* in soleus muscle, which is also upregulated in both NR groups compared to controls. However, the differences seen in INSRB, SLC2A1, SLC2A4, and myofiber type composition indicate a divergent response between NR(SGA) and NR(Non-SGA) within gastrocnemius muscle. This supports the fact that regardless of presenting a normal fetal weight, our NR(Non-SGA) group represents a unique phenotype, and is still unknown what metabolic alterations will be observed during adulthood as a consequence of this exclusive programming path.

Here we have also confirmed the relative resistance of soleus muscle to maternal NR. Soleus and gastrocnemius muscles are expected to have different metabolic characteristics, and the majority of muscles in the body are likely to have a phenotype that is closer to gastrocnemius. Hence, we interpret results found for gastrocnemius

muscle as more representative of whole-body skeletal muscle mass. Our results highlight the relevance of considering muscle-specific responses when trying to extrapolate results from a single muscle to the whole body.

2. Conclusions

In conclusion, we observed that our maternal NR model leads to differential effects on NR(SGA) and NR(Non-SGA) fetuses in terms of muscle growth and metabolism. While the observed results for NR(SGA) fetuses agree with previous observations in the literature, our results for NR(Non-SGA) indicate a unique metabolic programming effect. Our results are an initial indicator that having normal fetal weight after prenatal NR does not necessarily indicate an absence of molecular programming, which may impair metabolism during adulthood to an unknown extent. Future studies using isotopic tracers would provide essential information to evaluate dynamic processes, such as protein synthesis rate, in order to further understand the implications of our current results at a whole-body level. Additionally, characterization of specific markers in organs other than skeletal muscle would facilitate the understanding of our current findings in an interorgan cross-talk context. Further, understanding the maternal or placental adaptations that lead to the establishment of NR(Non-SGA) phenotype is of high interest to understand the observed fetal responses.

REFERENCES

Alessi, D. R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 15:6541-6551.

Allen, D. L., R. R. Roy, and V. R. Edgerton. 1999. Myonuclear domains in muscle adaptation and disease. Muscle Nerve 22:1350–1360.

Alexandriatos, N. and J. Bruinsma. 2012. World agriculture towards 2030/2050: the 2012 revision. ESA Working paper No. 12-03. Rome, FAO. 1-147.

Argilés, J. M., N. Campos, J. M. Lopez-Pedrosa, R. Rueda, and L. Rodriguez-Manas. 2016. Skeletal muscle regulates metabolism via interorgan crosstalk: roles in health and disease. J. Am. Med. Dir. Assoc. 17:789–796.

Atlantis, E., S. A. Martin, M. T. Haren, A. W. Taylor, and G. A. Wittert. 2009. Inverse associations between muscle mass, strength, and the metabolic syndrome. Metab. Clin. Exp. 58:1013–1022.

Baker, J., J. P. Liu, E. J. Robertson, and A. Efstratiadis. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75:73–82.

Barker, D., and P. M. Clark. 1997. Fetal undernutrition and disease in later life. Rev. Reprod. 2:105–112.

Barker, D. J., C. Osmond, P. Winter, B. Margetts, and S. J. Simmonds. 1989. Weight in infancy and death from ischaemic heart disease. Lancet 334:577–580.

Barry, J. S., and R. V. Anthony. 2008. The pregnant sheep as a model for human pregnancy. Theriogenology 69:55–67.

Bauman, D. E., and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63:1514–1529.

Belfiore, A., F. Frasca, G. Pandini, L. Sciacca, and R. Vigneri. 2009. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr. Rev. 30:586–623.

Belkacemi, L., C. H. Chen, M. G. Ross, and M. Desai. 2009. Increased placental apoptosis in maternal food restricted gestations: role of the Fas pathway. Placenta

30:739–751.

Bodine, S. C., T. N. Stitt, M. Gonzalez, W. O. Kline, G. L. Stover, R. Bauerlein, E. Zlotchenko, A. Scrimgeour, J. C. Lawrence, D. J. Glass, and G. D. Yancopoulos. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat. Cell Biol. 3:1014–1019.

Boucher, J., A. Kleinridders, and C. R. Kahn. 2014. Insulin receptor signaling in normal and insulin-resistant states. Cold Spring Harb. Perspect. Biol.6:a009191:1-23.

Brace, R. A. 1983. Blood volume and its measurement in the chronically catheterized sheep fetus. Am. J. Physiol. 244:H487–H494.

Brace, R. A. 1986. Fetal blood volume responses to acute fetal hypoxia. Am. J. Obstet. Gynecol. 155:889–893.

Braun, T. P., and D. L. Marks. 2015. The regulation of muscle mass by endogenous glucocorticoids. Front. Physiol. 6:1-12.

Brenner, B. M., D. L. Garcia, and S. Anderson. 1988. Glomeruli and blood pressure: less of one, more the other? Am. J. Hypertens. 1:335–347.

Brill, A., A. Torchinsky, H. Carp, and V. Toder. 1999. The role of apoptosis in normal and abnormal embryonic development. J. Assist. Reprod. Genet. 16:512–519.

Brown, L. D., and W. W. Hay Jr. 2016. Impact of placental insufficiency on fetal skeletal muscle growth. Mol. Cell. Endocrinol. 435:69–77.

Brown, L. D. 2014. Endocrine regulation of fetal skeletal muscle growth: impact on future metabolic health. J. Endocrinol. 221:R13–R29.

Brown, L.D., S. Thorn, M. C. O'Meara, J.R. Lavezzi, and P.J. Rozance. 2014. A physiological increase in insulin suppresses muscle-specific ubiquitin ligase gene activation in fetal sheep with sustained hypoglycemia. Physiol. Rep. 2:e12045:1-10.

Brown, M., J. Jansen, and D. Van Essen. 1976. Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. J. Physiol. 261:387–422.

Challis, J. R., L. Fraher, J. Oosterhuis, S. E. White, and A. D. Bocking. 1989. Fetal and maternal endocrine responses to prolonged reductions in uterine blood flow in pregnant sheep. Am. J. Obstet. Gynecol. 160:926–932.

Corcoran, M. P., S. Lamon-Fava, and R. A. Fielding. 2007. Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise. Am. J. Clin. Nutr. 85:662–677.

Cossu, G., and U. Borello. 1999. Wnt signaling and the activation of myogenesis in mammals. EMBO J. 18:6867–6872.

Costello, P. M., A. Rowlerson, N. A. Astaman, F. E. W. Anthony, A. A. Sayer, C. Cooper, M. A. Hanson, and L. R. Green. 2008. Peri-implantation and late gestation maternal undernutrition differentially affect fetal sheep skeletal muscle development. J. Physiol. (Lond.) 586:2371–2379.

De Blasio, M. J., K. L. Gatford, J. S. Robinson, and J. A. Owens. 2007. Placental restriction of fetal growth reduces size at birth and alters postnatal growth, feeding activity, and adiposity in the young lamb. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292:R875–R886.

DeFronzo, R. A., and D. Tripathy. 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 32 Suppl 2:S157–S163.

Desai, M., N. J. Crowther, A. Lucas, and C. N. Hales. 1996. Organ-selective growth in the offspring of protein-restricted mothers. Br. J. Nutr. 76:591–603.

Dimitriadis, G., P. Mitrou, V. Lambadiari, E. Maratou, and S. A. Raptis. 2011. Insulin effects in muscle and adipose tissue. Diabetes Res. Clin. Pract. 93:S52–S59.

Dominov, J. A., C. A. Houlihan-Kawamoto, C. J. Swap, and J. B. Miller. 2001. Pro- and anti-apoptotic members of the Bcl-2 family in skeletal muscle: a distinct role for Bcl-2 in later stages of myogenesis. Dev. Dyn. 220:18–26.

Du, M., J. Tong, J. Zhao, K. R. Underwood, M. Zhu, S. P. Ford, and P. W. Nathanielsz. 2010. Fetal programming of skeletal muscle development in ruminant animals. J. Anim. Sci. 88:E51–E60.

Dupont-Versteegden, E. E. 2006. Apoptosis in skeletal muscle and its relevance to atrophy. World J. Gastroenterol. 12:7463–7466.

Egerman, M. A., and D. J. Glass. 2014. Signaling pathways controlling skeletal muscle mass. Crit. Rev. Biochem. Mol. Biol. 49:59–68.

FAO, IFAD, UNICEF, WFP and WHO. 2017. The state of food security and nutrition in the world. Building climate resilience for peace and food security. Rome, FAO.1-117.

Fahey, A. J., J. M. Brameld, T. Parr, and P. J. Buttery. 2005. The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb. J. Anim. Sci. 83:2564–2571.

Fan, L., P. N. Hsieh, D. R. Sweet, and M. K. Jain. 2018. Krüppel-like factor 15: regulator of BCAA metabolism and circadian protein rhythmicity. Pharmacol. Res.130:123–126.

Fernandez-Twinn, D. S., and S. E. Ozanne. 2006. Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome. Physiol. Behav. 88:234–243.

Ferrannini, E., O. Bjorkman, G. A. Reichard, A. Pilo, M. Olsson, J. Wahren, and R. A. DeFronzo. 1985. The disposal of an oral glucose load in healthy subjects. A quantitative study. Diabetes 34:580–588.

Fidziañska, A. 1996. Apoptosis in human embryonic and diseased skeletal muscle. BAM-PADOVA- 6:261–264.

Finkelstein, D. I., P. Andrianakis, A. R. Luff, and D. W. Walker. 1992. Developmental changes in hindlimb muscles and diaphragm of sheep. Am. J. Physiol. 263:R900–R908.

Fisher, G., S. T. Windham, P. Griffin, J. L. Warren, B. A. Gower, and G. R. Hunter. 2017. Associations of human skeletal muscle fiber type and insulin sensitivity, blood lipids, and vascular hemodynamics in a cohort of premenopausal women. Eur. J. Appl. Physiol. 117:1413–1422.

Floyd, J., S. S. Fajans, J. W. Conn, R. F. Knopf, and J. Rull. 1966. Stimulation of insulin secretion by amino acids. J. Clin. Invest. 45:1487–1502.

Ford, S., B. Hess, M. Schwope, M. Nijland, J. Gilbert, K. Vonnahme, W. Means, H. Han, and P. Nathanielsz. 2007. Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. J. Anim. Sci. 85:1285–1294.

Fowden, A. L., P. Hughes, and R. S. Comline. 1989. The effects of insulin on the growth rate of the sheep fetus during late gestation. Q J Exp Physiol 74:703–714.

Fowden, A. L. 2003. The insulin-like growth factors and feto-placental growth. Placenta 24:803–812.

Gao, F., X. Hou, Y. Liu, S. Wu, and C. Ao. 2008. Effect of maternal under-nutrition during late pregnancy on lamb birth weight. Asian-Australas. J. Anim. Sci. 21:371–375.

Gao, J., J. Ren, E. A. Gulve, and J. O. Holloszy. 1994. Additive effect of contractions and insulin on GLUT-4 translocation into the sarcolemma. J. Appl. Physiol. 77:1597–1601.

Garber, A. J., I. Karl, and D. Kipnis. 1976. Alanine and glutamine synthesis and release from skeletal muscle. II. The precursor role of amino acids in alanine and glutamine synthesis. J. Biol. Chem. 251:836–843.

Gardner, D. S., K. Tingey, B. W. M. Van Bon, S. E. Ozanne, V. Wilson, J. Dandrea, D. H. Keisler, T. Stephenson, and M. E. Symonds. 2005. Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289:R947–R954.

Garg, M., M. Thamotharan, S. A. Oak, G. Pan, D. C. Maclaren, P. W. N. Lee, and S. U. Devaskar. 2009. Early exercise regimen improves insulin sensitivity in the intrauterine growth-restricted adult female rat offspring. Am. J. Physiol. Endocrinol. Metab. 296:E272–E281.

Garofano, A., P. Czernichow, and B. Breant. 1997. In utero undernutrition impairs rat beta-cell development. Diabetologia 40:1231–1234.

Gennser, G., P. Rymark, and P. E. Isberg. 1988. Low birth weight and risk of high blood pressure in adulthood. Br Med J (Clin Res Ed) 296:1498–500.

George, L. A., L. Zhang, N. Tuersunjiang, Y. Ma, N. M. Long, A. B. Uthlaut, D. T. Smith, P. W. Nathanielsz, and S. P. Ford. 2012. Early maternal undernutrition programs increased feed intake, altered glucose metabolism and insulin secretion, and liver function in aged female offspring. Am. J. Physiol. Regul. Integr. Comp. Physiol. 302:R795–R804.

Geremia, C., and S. Cianfarani. 2004. Insulin sensitivity in children born small for gestational age (SGA). Rev. Diabet. Stud. 1:58-65.

Gluckman, P. D., M. A. Hanson, and H. G. Spencer. 2005. Predictive adaptive responses and human evolution. Trends Ecol. Evol. (Amst.) 20:527–533.

Gluckman, P., M. Hanson, and T. Buklijas. 2010. A conceptual framework for the developmental origins of health and disease. J. Dev. Orig. Health Dis. 1:6–18.

Gokulakrishnan, G., I. J. Estrada, H. A. Sosa, and M. L. Fiorotto. 2012. In utero glucocorticoid exposure reduces fetal skeletal muscle mass in rats independent of effects on maternal nutrition. Am. J. Physiol. Regul. Integr. Comp. Physiol. 302:R1143–R1152.

Goldenberg, R. L., and S. P. Cliver. 1997. Small for gestational age and intrauterine growth restriction: definitions and standards. Clin. Obstet. Gynecol. 40:704–714.

Gray, S., B. Wang, Y. Orihuela, E.-G. Hong, S. Fisch, S. Haldar, G. W. Cline, J. K. Kim, O. D. Peroni, B. B. Kahn, and M. K. Jain. 2007. Regulation of gluconeogenesis by Krüppel-like factor 15. Cell Metab. 5:305–12.

Haldar, S. M., D. Jeyaraj, P. Anand, H. Zhu, Y. Lu, D. A. Prosdocimo, B. Eapen, D. Kawanami, M. Okutsu, L. Brotto, H. Fujioka, J. Kerner, M. G. Rosca, O. P. McGuinness, R. J. Snow, A. P. Russell, A. N. Gerber, X. Bai, Z. Yan, T. M. Nosek, M. Brotto, C. L. Hoppel, and M. K. Jain. 2012. Kruppel-like factor 15 regulates skeletal muscle lipid flux and exercise adaptation. Proc. Natl. Acad. Sci. U.S.A. 109:6739–6744.

Hales, C. N., and D. J. Barker. 1992. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35:595–601.

He, J., S. Watkins, and D. E. Kelley. 2001. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. Diabetes 50:817–823.

Hellman, B., E. Gylfe, P. Bergsten, E. Grapengiesser, P. E. Lund, A. Berts, A. Tengholm, D. G. Pipeleers, and Z. Ling. 1994. Glucose induces oscillatory Ca2+

signalling and insulin release in human pancreatic beta cells. Diabetologia 37 Suppl 2:S11–S20.

Hikida, R. S., S. Van Nostran, J. D. Murray, R. S. Staron, S. E. Gordon, and W. J. Kraemer. 1997. Myonuclear loss in atrophied soleus muscle fibers. Anat. Rec. 247:350–354.

Howden, S., S. Crimp, and C. Stokes. 2008. Climate change and Australian livestock systems: impacts, research and policy issues. An. Prod. Sci. 48:780–788.

Hyatt, M.A., D.S. Gardner, S. Sebert, V. Wilson, N. Davidson, Y. Nigmatullina, L.L.Y Chan, H. Budge, and M.E. Symonds. 2011. Suboptimal maternal nutrition, during early fetal liver development, promotes lipid accumulation in the liver of obese offspring. Reproduction 141:119-126.

Hyatt, M. A., H. Budge, D. Walker, T. Stephenson, and M. E. Symonds. 2007. Effects of maternal parity and late gestational nutrition on mRNA abundance for growth factors in the liver of postnatal sheep. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292:R1934–R1942.

Ibáñez, L., K. Ong, D. B. Dunger, and F. de Zegher. 2006. Early development of adiposity and insulin resistance after catch-up weight gain in small-for-gestational-age children. J. Clin. Endocrinol. Metab. 91:2153–2158.

James, D. E., R. Brown, J. Navarro, and P. F. Pilch. 1988. Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. Nature 333:183.

Janssen, I., S. B. Heymsfield, Z. Wang, and R. Ross. 2000. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. J. Appl. Physiol. 89:81–88.

Jones, N. C., Y. V. Fedorov, R. S. Rosenthal, and B. B. Olwin. 2001. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. J. Cell. Physiol. 186:104–115.

Joulia, D., H. Bernardi, V. Garandel, F. Rabenoelina, B. Vernus, and G. Cabello. 2003. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Exp. Cell Res. 286:263–75.

Kalbe, C., J. Bérard, M. Porm, C. Rehfeldt, and G. Bee. 2013. Maternal L-arginine supplementation during early gestation affects foetal skeletal myogenesis in pigs. Livest. Sci. 157:322–329.

Kalogeropoulou, D., L. Lafave, K. Schweim, M. C. Gannon, and F. Q. Nuttall. 2008. Leucine, when ingested with glucose, synergistically stimulates insulin secretion and lowers blood glucose. Metab. Clin. Exp. 57:1747–1752.

Kensara, O. A., S. A. Wootton, D. I. Phillips, M. Patel, A. A. Jackson, and M. Elia. 2005. Fetal programming of body composition: relation between birth weight and body composition measured with dual-energy X-ray absorptiometry and anthropometric methods in older Englishmen. Am. J. Clin. Nutr. 82:980–987.

Kim, D.H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110:163–175.

Kim, J. K., J. J. Fillmore, Y. Chen, C. Yu, I. K. Moore, M. Pypaert, E. P. Lutz, Y. Kako, W. Velez-Carrasco, I. J. Goldberg, J. L. Breslow, and G. I. Shulman. 2001. Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. Proc. Natl. Acad. Sci. U.S.A. 98:7522–7527.

Kohn, A. D., S. A. Summers, M. J. Birnbaum, and R. A. Roth. 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J. Biol. Chem. 271:31372–31378.

Kwon, H., S. P. Ford, F. W. Bazer, T. E. Spencer, P. W. Nathanielsz, M. J. Nijland, B. W. Hess, and G. Wu. 2004. Maternal nutrient restriction reduces concentrations of amino acids and polyamines in ovine maternal and fetal plasma and fetal fluids. Biol. Reprod. 71:901–908.

Lassala, A., F. W. Bazer, T. A. Cudd, S. Datta, D. H. Keisler, M. C. Satterfield, T. E. Spencer, and G. Wu. 2010. Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. J. Nutr. 140:1242–1248.

Lea, R. G., L. Andrade, M. Rae, L. T. Hannah, C. Kyle, J. F. Murray, S. Rhind, and D. Miller. 2006. Effects of maternal undernutrition during early pregnancy on apoptosis regulators in the ovine fetal ovary. Reproduction 131:113–124.

Lie, S., J. L. Morrison, O. Williams-Wyss, C. M. Suter, D. T. Humphreys, S. E. Ozanne, S. Zhang, S. M. MacLaughlin, D. O. Kleemann, S. K. Walker, et al. 2014. Periconceptional undernutrition programs changes in insulin-signaling molecules and microRNAs in skeletal muscle in singleton and twin fetal sheep. Biol. Reprod. 90:1-10.

Lillioja, S., A. A. Young, C. L. Culter, J. L. Ivy, W. G. Abbott, J. K. Zawadzki, H. Yki-Järvinen, L. Christin, T. W. Secomb, and C. Bogardus. 1987. Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. J.

Clin. Invest. 80:415–424.

Limesand, S. W., P. J. Rozance, G. O. Zerbe, J. C. Hutton, and W. W. Hay. 2006. Attenuated insulin release and storage in fetal sheep pancreatic islets with intrauterine growth restriction. Endocrinology 147:1488–1497.

Lin, G., X. Wang, G. Wu, C. Feng, H. Zhou, D. Li, and J. Wang. 2014. Improving amino acid nutrition to prevent intrauterine growth restriction in mammals. Amino Acids 46:1605–1623.

Lloyd, L. J., T. Foster, P. Rhodes, S. M. Rhind, and D. S. Gardner. 2012. Protein-energy malnutrition during early gestation in sheep blunts fetal renal vascular and nephron development and compromises adult renal function. J. Physiol. 590:377–393.

Lokireddy, S., V. Mouly, G. Butler-Browne, P. D. Gluckman, M. Sharma, R. Kambadur, and C. McFarlane. 2011. Myostatin promotes the wasting of human myoblast cultures through promoting ubiquitin-proteasome pathway-mediated loss of sarcomeric proteins. Am. J. Physiol. Cell Physiol. 301:C1316–C1324.

Long, J. H., V. A. Lira, Q. A. Soltow, J. L. Betters, J. E. Sellman, and D. S. Criswell. 2006. Arginine supplementation induces myoblast fusion via augmentation of nitric oxide production. J. Muscle. Res. Cell. Motil. 27:577–584.

Manning, B. D., and A. Toker. 2017. AKT/PKB Signaling: Navigating the Network, Cell. 169:381-405.

Manjarin, R., D. A. Columbus, J. Solis, A. D. Hernandez-García, A. Suryawan, H. V. Nguyen, M. M. McGuckin, R. T. Jimenez, M. L. Fiorotto, and T. A. Davis. 2018. Short- and long-term effects of leucine and branched-chain amino acids supplementation of a protein- and energy-reduced diet on muscle protein metabolism in neonatal pigs. Amino Acids 50:943-959.

Maltin, C. A. 2008. Muscle development and obesity: is there a relationship? Organogenesis 4:158–169.

Marliss, E. B., T. T. Aoki, T. Pozefsky, A. S. Most, and G. F. Cahill. 1971. Muscle and splanchnic glutamine and glutamate metabolism in postabsorptive starved man. J. Clin. Invest. 50:814–817.

McCoard, S. A., W. C. McNabb, M. J. Birtles, P. M. Harris, S. N. McCutcheon, and S. W. Peterson. 2001. Immunohistochemical detection of myogenic cells in muscles of fetal and neonatal lambs. Cells Tissues Organs (Print) 169:21–33.

McMillen, I. C., and J. S. Robinson. 2005. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol. Rev. 85:571–633.

Menconi, M., M. Fareed, P. O'Neal, V. Poylin, W. Wei, and P.-O. Hasselgren. 2007. Role of glucocorticoids in the molecular regulation of muscle wasting. Crit. Care Med. 35:S602–S608.

Mora, S., P. Kaliman, J. Chillarón, X. Testar, M. Palacín, and A. Zorzano. 1995. Insulin and insulin-like growth factor I (IGF-I) stimulate GLUT4 glucose transporter translocation in Xenopus oocytes. Biochem. J. 311(Pt 1):59–65.

Morissette, M. R., S. A. Cook, C. Buranasombati, M. A. Rosenberg, and A. Rosenzweig. 2009. Myostatin inhibits IGF-I-induced myotube hypertrophy through Akt. Am. J. Physiol. Cell Physiol. 297:C1124–C1132.

Morrison, J. L., J. A. Duffield, B. S. Muhlhausler, S. Gentili, and I. C. McMillen. 2010. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. Pediatr. Nephrol. 25:669–677.

Morrison, J. L. 2008. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. Clin. Exp. Pharmacol. Physiol. 35:730–743.

Nardone, A., B. Ronchi, N. Lacetera, M. S. Ranieri, and U. Bernabucci. 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livest. Sci. 130:57–69.

Newsholme, P., L. Brennan, and K. Bender. 2006. Amino acid metabolism, beta-cell function, and diabetes. Diabetes 55:S39–S47.

Nicholson, D. W. 1999. Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ. 6:1028–1042.

Nyholm, B., Z. Qu, A. Kaal, S. B. Pedersen, C. H. Gravholt, J. L. Andersen, B. Saltin, and O. Schmitz. 1997. Evidence of an increased number of type IIb muscle fibers in insulin-resistant first-degree relatives of patients with NIDDM. Diabetes 46:1822–1828.

Ogata, T., and M. Mori. 1964. Histochemical study of oxidative enzymes in invertebrate muscles. J. Histochem. Cytochem. 12:183–187.

Osgerby, J. C., D. C. Wathes, D. Howard, and T. S. Gadd. 2002. The effect of maternal undernutrition on ovine fetal growth. J. Endocrinol. 173:131–141.

Ozanne, S. E., and C. N. Hales. 1999. The long-term consequences of intra-uterine protein malnutrition for glucose metabolism. Proc. Nutr. Soc. 58:615–619.

Ozanne, S. E., C. L. Wang, N. Coleman, and G. D. Smith. 1996. Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. Am. J. Physiol. 271:E1128–134.

Peinado, B., R. Latorre, J. M. Váquez-Autón, A. Poto, G. Ramírez, O. López-Albors, F. Moreno, and F. Gil. 2004. Histochemical skeletal muscle fibre types in the sheep. Anat Histol Embryol 33:236–243.

Pérez, R., I. Tupac-Yupanqui, and S. Dunner. 2008. Evaluation of suitable reference genes for gene expression studies in bovine muscular tissue. BMC Mol. Biol. 9:1-6.

Pham, T. D., N. K. MacLennan, C. T. Chiu, G. S. Laksana, J. L. Hsu, and R. H. Lane. 2003. Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. Am. J. Physiol. Regul. Integr. Comp. Physiol. 285:R962–R970.

Picard, B., L. Lefaucheur, C. Berri, and M. J. Duclos. 2002. Muscle fibre ontogenesis in farm animal species. Reprod. Nutr. Dev. 42:415–431.

Quigley, S. P., D. O. Kleemann, M. A. Kakar, J. A. Owens, G. S. Nattrass, S. Maddocks, and S. K. Walker. 2005. Myogenesis in sheep is altered by maternal feed intake during

the peri-conception period. Anim. Reprod. Sci. 87:241–251.

Rattray, P., and J. Joyce. 1976. Utilisation of metabolizable energy for fat and protein deposition in sheep. New Zeal. J. Agr. Res.19:299–305.

Redfern, P. A. 1970. Neuromuscular transmission in new-born rats. J. Physiol. 209:701-709.

Rich-Edwards, J. W., G. A. Colditz, M. J. Stampfer, W. C. Willett, M. W. Gillman, C. H. Hennekens, F. E. Speizer, and J. E. Manson. 1999. Birthweight and the risk for type 2 diabetes mellitus in adult women. Ann. Intern. Med. 130:278–284.

Rodriguez, J., B. Vernus, I. Chelh, I. Cassar-Malek, J.-C. Gabillard, A. H. Sassi, I. Seiliez, B. Picard, and A. Bonnieu. 2014. Myostatin and the skeletal muscle atrophy and hypertrophy signaling pathways. Cell. Mol. Life Sci.71:4361–4371.

Rommel, C., S. C. Bodine, B. A. Clarke, R. Rossman, L. Nunez, T. N. Stitt, G. D. Yancopoulos, and D. J. Glass. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat. Cell Biol. 3:1009–1013.

Rosenblatt, J. D., D. Yong, and D. J. Parry. 1994. Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle Nerve 17:608–613.

Rozance, P., S.W. Limesand, J.S. Barris, L.D. Brown, S.R. Thorn, D. LoTurco, T.R.H. Regnault, J.E. Friedman, and W. Hay. 2007. Chronic late-gestation hypoglycemia upregulates hepatic PEPCK associated with increased PGC1α mRNA and phosphorylated CREB in fetal sheep. Am. J. Endocrinol. Metab. 294:E365-E370.

Rudar, M., M. L. Fiorotto, and T. A. Davis. 2019. Regulation of Muscle Growth in Early Postnatal Life in a Swine Model. Annu. Rev. Anim. Biosci. 7:309–335.

Sandoval, C., C.A. Lambo, and M.C. Satterfield. 2018. Redirecting Fetal Programming: Evidence of Interventions that May be a Tool for Health. OBM Genetics 2:1-19.

Sandri, M., and U. Carraro. 1999. Apoptosis of skeletal muscles during development and disease. Int. J. Biochem. Cell Biol. 31:1373–1390.

Sandri, M., A. H. El Meslemani, C. Sandri, P. Schjerling, K. Vissing, J. L. Andersen, K. Rossini, U. Carraro, and C. Angelini. 2001. Caspase 3 expression correlates with skeletal muscle apoptosis in Duchenne and facioscapulo human muscular dystrophy. A potential target for pharmacological treatment? J. Neuropathol. Exp. Neurol. 60:302–312.

Sandri, M. 2013. Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. Int. J. Biochem. Cell Biol. 45:2121–2129.

Santalucia, T., M. Camps, A. Castello, P. Munoz, A. Nuel, X. Testar, M. Palacin, and A. Zorzano. 1992. Developmental regulation of GLUT-1 (erythroid/Hep G2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle, and brown adipose tissue. Endocrinology 130:837–846.

Satterfield, M. C., F. W. Bazer, T. E. Spencer, and G. Wu. 2009. Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. J. Nutr.140:251–258.

Satterfield, M. C., K. A. Dunlap, D. H. Keisler, F. W. Bazer, and G. Wu. 2013. Arginine nutrition and fetal brown adipose tissue development in nutrient-restricted sheep. Amino Acids 45:489–499.

Satterfield, M. C., K. Hayashi, G. Song, S. G. Black, F. W. Bazer, and T. E. Spencer. 2008. Progesterone regulates FGF10, MET, IGFBP1, and IGFBP3 in the endometrium of the ovine uterus. Biol. Reprod. 79:1226–1236.

Satterfield, M. C., and G. Wu. 2011. Brown adipose tissue growth and development: significance and nutritional regulation. Front. Biosci. 16:1589–1608.

Saxton, R.A., D.M. Sabatini. 2017. mTOR signaling in growth, metabolism, and disease. Cell 168:960-976.

Schakman, O., S. Kalista, C. Barbé, A. Loumaye, and J. P. Thissen. 2013. Glucocorticoid-induced skeletal muscle atrophy. Int. J. Biochem. Cell Biol. 45:2163–172.

Scheepers, A., H. Joost, and A. Schürmann. 2004. The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. J. Parenter. Enteral Nutr. 28:364-371.

Schiaffino, S., and C. Mammucari. 2011. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. Skelet. Muscle 1:1-14.

Schmidt, H. H., T. D. Warner, K. Ishii, H. Sheng, and F. Murad. 1992. Insulin secretion from pancreatic B cells caused by L-arginine-derived nitrogen oxides. Science 255:721–723.

Shimizu, N., N. Yoshikawa, N. Ito, T. Maruyama, Y. Suzuki, S. Takeda, J. Nakae, Y. Tagata, S. Nishitani, K. Takehana, M. Sano, K. Fukuda, M. Suematsu, C. Morimoto, and

H. Tanaka. 2011. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell Metab. 13:170–182.

Shukla, P., S. Ghatta. D. Dubey, C. O. Lemley, M. L. Johnson, A. Modgil, K. Vonnahme, J. S. Caton, L. P. Reynolds, C. Sun, and S. O'Rourke. 2014. Maternal nutrient restriction during pregnancy impairs an endothelium-derived hyperpolarizing factor-like pathway in sheep fetal coronary arteries. Am. J. Physiol. Heart Circ. Physiol. 307:H134-H142.

Spencer, T. E., F. F. Bartol, F. W. Bazer, G. A. Johnson, and M. M. Joyce. 1999. Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. Biol. Reprod. 60:241-250.

Stewart, C.E.H., Rittweger, J. 2006. Adaptive processes in skeletal muscle: Molecular regulators and genetic influences. J. Musculoskelet. Neuronal Interact. 6:73-86.

Stuart, C. A., G. Wen, W. C. Gustafson, and E. A. Thompson. 2000. Comparison of GLUT1, GLUT3, and GLUT4 mRNA and the subcellular distribution of their proteins in normal human muscle. Metab. Clin. Exp. 49:1604–1609.

Suryawan, A., A. S. Jeyapalan, R. A. Orellana, F. A. Wilson, H. V. Nguyen, and T. A. Davis. 2008. Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by

enhancing mTORC1 activation. Am. J. Physiol. Endocrinol. Metab. 295:E868–E875.

Symonds, M. E., S. P. Sebert, M. A. Hyatt, and H. Budge. 2009. Nutritional programming of the metabolic syndrome. Nat. Rev. Endocrinol. 5:604–610.

Tafti, S. A., C.C. Nast, M. Desai, K.E. Amaya, M.G. Ross, and T. R. Magee. 2011. Maternal undernutrition upregulates apoptosis in offspring nephrogenesis. J. Devep. Orig. Health Dis. 2:226-235.

Tan, B., Y. Yin, Z. Liu, X. Li, H. Xu, X. Kong, R. Huang, W. Tang, I. Shinzato, S. B. Smith, and G. Wu. 2009. Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. Amino Acids 37:169–175.

Taniguchi, C., B. Emanuelli, and C. R. Kahn. 2006. Critical nodes in signaling pathways: insights into insulin action. Nat. Rev. 7:85-96.

Takeuchi, Y., N. Yahagi, Y. Aita, Y. Murayama, Y. Sawada, X. Piao, N. Toya, Y. Oya, A. Shikama, A. Tajarada, Y. Masuda, M. Nishi, et al. KLF15 enables rapid switching between lipogenesis and gluconeogenesis during fasting. 2016. Cell Rep. 16:2373-2386.

Todd, S. E., M. H. Oliver, A. L. Jaquiery, F. H. Bloomfield, and J. E. Harding. 2009. Periconceptional undernutrition of ewes impairs glucose tolerance in their adult offspring. Pediatr. Res. 65:409–413.

Trendelenburg, A. U., A. Meyer, D. Rohner, J. Boyle, S. Hatakeyama, and D. J. Glass. 2009. Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. Am. J. Physiol. Cell Physiol. 296:C1258–1270.

UNICEF and WHO. 2004. Low birthweight: Country, regional and global estimates. UNICEF, New York.1-27.

Vaughan, O. R., M. J. De Blasio, and A. L. Fowden. 2018. Ovine uteroplacental and fetal metabolism during and after fetal cortisol overexposure in late gestation. Am. J. Physiol. Regul. Integr. Comp. Physiol. 314:R791-R801.

Verhaeghe, J., R. Van Bree, E. Van Herck, J. Laureys, R. Bouillon, and F. A. Van Assche. 1993. C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in umbilical cord serum: correlations with birth weight. Am. J. Obstet. Gynecol. 169:89-97.

Vonnahme, K.A., B.W. Hess, T.R. Hansen, R.J. McCormick, D.C. Rule, G.E. Moss, W.J. Murdoch, M.J. Nijland, D.C. Skinner, P.W. Nathanielsz, and S. P. Ford. 2003.

Maternal undernutrition from early-to-mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. Biol. Reprod. 69:133-140.

Vonnahme, K. A., J. S. Luther, L. P. Reynolds, C. J. Hammer, D. B. Carlson, D. A. Redmer, and J. S. Caton. 2010. Impacts of maternal selenium and nutritional level on growth, adiposity, and glucose tolerance in female offspring in sheep. Domest. Anim. Endocrinol. 39:240–248.

Wali, J. A., H. A. de Boo, J. G. B, Derraik, H. H. Phua, M. H. Oliver, F. H. Bloomfield, and J. E. Harding. 2012. Weekly intra-amniotic IGF-1 treatment increases growth of growth-restricted ovine fetuses and up-regulates placental amino acid transporters. PloS ONE. 7:e37899:1-13.

Walther, G., E. Post, P. Convey, A. Menzel, C. Parmesan, T. J.C. Beebee, J. Fromentin, O. Hoegh-Guldberg, and F. Bairlein. 2002. Ecological responses to recent climate change. Nature 416:389-395.

Wang, X., J. W. Frank, J. Xu, K. A. Dunlap, M. C. Satterfield, R. C. Burghardt, J. J. Romero, T. R. Hansen, G. Wu, and F. W. Bazer. 2014. Functional role of arginine during the peri-implantation period of pregnancy. II. Consequences of loss of function of

nitric oxide synthase *NOS3* mRNA in ovine conceptus trophectoderm. Biol. Reprod. 91:1-10.

Ward, S. S., and N. C. Stickland. 1991. Why are slow and fast muscles differentially affected during prenatal undernutrition? Muscle Nerve 14:259–267.

Warram, J. H., B. C. Martin, A. S. Krolewski, J. S. Soeldner, and C. R. Kahn. 1990. Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. Ann. Inter. Med. 113:909-915.

Wei, C., L. Li, H. Su, L. Xu, J. Lu, L. Zhang, W. Liu, H. Ren, L. Du. 2014. Identification of the crucial molecular events during the large-scale myoblast fusion in sheep. Physiol. Genomics. 46:429-440.

Whitman, S. A., M. J. Wacker, S. R. Richmond, and M. P. Godard. 2005. Contributions of the ubiquitin-proteasome pathway and apoptosis to human skeletal muscle wasting with age. Pflugers Arch. 450:437–446.

Wigmore, P. M., and N. C. Stickland. 1983. Muscle development in large and small pig fetuses. J. Anat. 137 (Pt 2):235–245.

Wray, C. J., J. M. V. Mammen, D. D. Hershko, and P.-O. Hasselgren. 2003. Sepsis upregulates the gene expression of multiple ubiquitin ligases in skeletal muscle. Int. J. Biochem. Cell Biol. 35:698–705.

Wu, G. 2016. Dietary protein intake and human health. Food Funct. 7:1251-1265.

Wu, G. 2013a. Regulation of amino acid metabolism. In: Wu, G. Amino acids biochemistry and nutrition. CRC Press, Boca Raton, FL. 303-337.

Wu, G. 2013b. Physiological functions of amino acids. In: Wu, G. Amino acids biochemistry and nutrition. CRC Press, Boca Raton, FL. 339-388.

Wu, G., F. W. Bazer, T. A. Davis, S. W. Kim, P. Li, J. Marc Rhoads, M. Carey Satterfield, S. B. Smith, T. E. Spencer, and Y. Yin. 2009. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168.

Wu, G., F. W. Bazer, J. M. Wallace, and T. E. Spencer. 2006. Board-invited review: intrauterine growth retardation: implications for the animal sciences. J. Anim. Sci. 84:2316–2337.

Yamamoto, J., Y. Ikeda, H. Iguchi, T. Fujino, T. Tanaka, H. Asaba, S. Iwasaki, R. X. Ioka, I. W. Kaneko, K. Magoori, S. Takahashi, T. Mori, H. Sakaue, T. Kodama, M.

Yanagisawa, T. T. Yamamoto, S. Ito, and J. Sakai. 2004. A Kruppel-like factor KLF15 contributes fasting-induced transcriptional activation of mitochondrial acetyl-CoA synthetase gene AceCS2. J. Biol. Chem. 279:16954–16962.

Yao, K., Y. Yin, W. Chu, Z. Liu, D. Deng, T. Li, R. Huang, J. Zhang, B. Tan, W. Wang, and G. Wu. 2008. Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. J. Nutr. 138:867-872.

Yoon, M. 2017. mTOR as a key regulator in maintaining skeletal muscle mass. Front. Physiol. 8:1-9.

Yuan, Y., Y. Xu, J. Xu, B. Liang, X. Cai, C. Zhu, L. Wang, S. Wang, X. Zhu, P. Gao, X. Wang, Y. Zhang, Q. Jiang, and G. Shu. 2017. Succinate promotes skeletal muscle protein synthesis via Erk1/2 signaling pathway. Mol. Med. Rep. 16:7661-7366.

Zhu, M. J., S. P. Ford, W. J. Means, B. W. Hess, P. W. Nathanielsz, and M. Du. 2006. Maternal nutrient restriction affects properties of skeletal muscle in offspring. J. Physiol. (Lond.). 575:241–50.

Zhu, M.-J., S. P. Ford, P. W. Nathanielsz, and M. Du. 2004. Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. Biol. Reprod. 71:1968–

1973.

Zorzano, A., M. Palacín, and A. Gumà. 2005. Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. Acta Physiol. Scand. 183:43–58.